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Molecular Biology and Genetics of Mycoplasmas (*Mollicutes*)

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INTRODUCTION

The special appeal in studying cell biology of mycoplasmas stems from the fact that they are the smallest and simplest self-replicating procaryotes. Mycoplasmas lack a cell wall and are bounded by a plasma membrane only. Their genome is the smallest recorded in living cells. All the mycoplasmas included in the Division *Tenericutes*, class *Mollicutes* (188) are most prevalent parasites of man, animals, arthropods, and plants and many cause disease.

The relative ease by which mycoplasma membranes can be isolated and the ability to manipulate their composition in a controlled manner have made them most useful tools for studying basic principles in membrane structure and function. The subject of mycoplasma membranes has been rather comprehensively discussed in a series of reviews published during the last decade (172, 217–219, 221, 246). Research in this field now appears to have slowed down, but recent advances in molecular genetics of mycoplasmas can be expected to boost membrane studies by providing new genetic tools which are particularly effective in research of membrane biosynthesis.

Compared with the impressive developments made in mycoplasma membrane research, mycoplasma genetics has remained undeveloped until recently. Thus, the section covering mycoplasma genetics in the last comprehensive review on mycoplasma biology (218) occupied less than 1/30th the space devoted to membranes. The major reason for this lag is the inadequacy of the classical genetic techniques when applied to mycoplasmas. Thus, dependence of mycoplasmas on highly complex growth media and their poorly defined metabolic pathways have hampered the selection of auxotropic mutants. The introduction of the powerful methods of recombinant deoxyribonucleic acid (DNA) technology to mycoplasma genetics has opened the way to isolate and characterize specific DNA segments of their small genome. It appears now that many of the current studies on mycoplasma biology have been diverted from the membrane to the genome, extrachromosomal elements, and ribosomes.

Analysis of the structure, physical properties, and expression of the mycoplasma genome has taken several directions. One direction is concerned with the nature of genes providing the minimal requirements for the existence of the smallest free-living organisms. Another direction is to use the structural and organizational characteristics of mycoplasma genes, particularly ribosomal ribonucleic acid (rRNA) genes, as phylogenetic markers to find out the place of mollicutes in the general evolutionary scheme. Still another line of research, of a more applied nature, focuses on the use of cloned mycoplasma genes as probes in diagnosis of mycoplasma infections and as tools in production of vaccines made of specific immunogens. The impact of the introduction of genetic tools to mycoplasma classification has also been rather impressive as it helps to overcome the great difficulties encountered in classifying the plethora of new mollicutes recently isolated from insects, plants, and animals. It will be clear to the reader of this review that information on the molecular biology and genetics of mycoplasmas is still fragmentary and is a far cry from that known for *Escherichia coli*. Yet the recent upsurge of interest in mycoplasma genetics makes it necessary to sum up the knowledge accumulated so far and try and outline new avenues for research.

The last extensive reviews on the molecular biology and genetics of mycoplasmas were written about 8 years ago (218, 261). The reader who seeks general information on mycoplasmas is referred to the three volumes of *The Mycoplasmas*, published in 1979 (16, 282, 287). More recent information on all aspects of mycoplasmology can be found in the proceedings of meetings of the International Organization for Mycoplasmology (17, 135, 226) and of other meetings dealing with more specific topics (26, 171, 220). Several recent reviews on plant and insect mycoplasmas are also available (23, 48, 286). An up-to-date picture of research on mycoplasma pathogenicity and mycoplasma-host cell interactions can be found in the latest volume of *The Mycoplasmas* (224). A comprehensive treatment of current techniques for handling mycoplasmas is presented in two volumes of *Methods in Mycoplasmology* (232, 280), and a detailed description of the various taxa and species comprising the class *Mollicutes* is given in the recent edition of *Bergey's Manual of Systematic Bacteriology* (139).

The trivial name mycoplasmas will be used in this review rather loosely to denote any species included in the class *Mollicutes*, whereas the trivial names acholeplasmas, ureaplasmas, anaeroplasmas, and spiroplasmas will be used when reference is made specifically to members of the corresponding genus. It has been suggested (269) that mollicutes be used as a trivial name for any member of the class. This has the advantage of keeping the trivial term mycoplasmas for *Mycoplasma* species only. In this review the terms mycoplasmas and mollicutes will be used interchangeably, dictated by convenience. For easy reference and orientation the current classification and major distinguishing properties of mollicutes are summarized in Table 1.

GENOME STRUCTURE

Size

The circular double-stranded genome of *Mollicutes* is distinguished by its minute size and low guanine-plus-cytosine (G+C) content. According to genome size all the mollicutes examined so far fall into two clusters: one composed of *Mycoplasma* and *Ureaplasma* species with a genome of about 500 megadaltons (MDa), and the other composed of *Acholeplasma*, *Spiroplasma*, and *Anaeroplasma* species with a genome about twice as large (for references, see reference 225). The gap in genome size between the two clusters, used by Morowitz and Wallace (179) as a basis for an evolutionary scheme (see Phylogeny section) has not yet been filled by mollicutes with intermediate genome sizes. However, genome size data are available only for a minority of established species, so that the existence of mollicutes with genome sizes between 500 and 1,000 MDa cannot be ruled out. Moreover, as stressed by Bove (22), published genome size values should be reconsidered, as in most cases determinations were based on renaturation kinetics (11) which are affected by the G+C content of the DNA. This factor was not usually taken into consideration in previous genome size measurements. Nevertheless, since the G+C content of mycoplasmal DNA falls, with very few exceptions, within a rather narrow range (25 to 34 mol%), the conclusions derived from renaturation kinetics as to the relative genome sizes appear to hold. Thus, spiroplasmas with a 26 mol% G+C genome were found by Bove (22) to have genomes twice as large as those of *Mycoplasma* species with the same G+C content, while spiroplasmas with a G+C content of 30 ± 1 mol% exhibited the same genome size as *Acholeplasma* species with a similar G+C content. The genome size data obtained by renaturation kinetics are supported by contour-length mea-

TABLE 1. Major characteristics of members of the class *Mollicutes*[a]

Classification	No. of established species	Genome size (MDa)	Sterol requirement	Special features	Habitat
Mycoplasmataceae					
Mycoplasma	Over 70	ca. 500	+		Humans and animals
Ureaplasma	2[b]	ca. 500	+	Urease activity	Humans and animals
Acholeplasmataceae					
Acholeplasma	10	ca. 1,000	−		Humans, animals, and plant surfaces
Spiroplasmataceae					
Spiroplasma	4[c]	ca. 1,000	+	Helical and motile filaments	Arthropods and plants
Anaeroplasmataceae[d]					
Anaeroplasma	2	ca. 1,000 (?)	Some + Some −	Strict anaerobes	Rumens of cattle and sheep

[a] The class *Mollicutes* is currently the only class in the Division *Tenericutes* and contains two orders: *Mycoplasmatales* and *Acholeplasmatales*. Each of the orders contains a single family, *Mycoplasmataceae* and *Acholeplasmataceae*, respectively (139).

[b] The marked genetic heterogeneity of ureaplasmas isolated from many animal species indicates that more species will be established in the future (112).

[c] Over 20 different serogroups of spiroplasmas isolated from arthropods and plants have already been recognized (23). Hence, the number of established species must represent a minute fraction of the spiroplasma species existing in nature.

[d] According to DNA hybridization, Stephens et al. (266) propose the establishment of a family *Anaeroplasmataceae* including two genera (according to sterol requirement) and five species. The proposal has yet to be approved.

surements of genomes spread on electron microscopical grids (2, 21, 50, 52, 178, 277).

Genomes of about 1,000 MDa appear to be rather rare in procaryotes. They were found in *Haemophilus influenzae*, two *Neisseria* species (12), a methanogenic archaebacterium (174), and the wall-less archaebacterium *Thermoplasma acidophilum* (40). However, not all archaebacteria are characterized by small genomes. Thus, *Halobacterium halobium* has a genome of 2,700 MDa (114). The genome size of *Rickettsia* species is approximately 1,000 MDa (191), and that of *Chlamydia trachomatis* has been estimated to be 660 MDa (253). Hence, 500-MDa genomes appear to be limited to *Mycoplasma* and *Ureaplasma* species.

The 500-MDa genome represents the smallest genome of a self-replicating organism. Mycoplasma cells are therefore considered by Morowitz and colleagues (176, 177, 179) to represent minimum living systems, since their coding capacity is limited to less than 700 different proteins, only twice the calculated number of proteins essential for the functioning of the smallest hypothetical cell designed according to the conventional results of molecular biology (176, 177). Recent data support the low coding capacity of mycoplasmas, by showing in two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) the presence of about 350 different polypeptides in *Mycoplasma capricolum* cells, compared with over 1,100 polypeptides detected under the same experimental conditions in *E. coli* and *Bacillus subtilis* cells (129). When the number of *M. capricolum* polypeptides is combined with about 50 genes coding for rRNA and transfer RNA (tRNA) species in this organism (6, 250, 255) one may conclude that about 400 genes are expressed, and their products can be detected in *M. capricolum*. This is not far from the 640 genes calculated by Morowitz (176, 178) for this organism, assuming an average molecular weight of 40,000 for a protein and discounting the existence of noncoding DNA regions in the flanking sequences of structural genes. It thus appears that the 2D-PAGE analysis of Kawauchi et al. (129) reveals the products of the great majority of the *M. capricolum* genes. This leads us to the recent proposal by Morowitz (177) to launch an international effort to test the dogma of the completeness of molecular biology by total deciphering of the machinery of a living cell. The task of complete sequencing of a mycoplasma genome of about 700 kilobase pairs (kbp) is within the range of the possible, since the 172-kbp genome of the Epstein-Barr virus has already been sequenced. By selecting a species with a very low G+C genome such as *M. capricolum*, it should be possible to uniquely read from the code to the amino acid sequence. In general, the first seven or eight N-terminal amino acids will locate the protein on the genome, and the remainder can be read out. A thorough study of the ultrastructure, metabolic maps, and transport systems should also be undertaken, with the final aim of assigning coding space to structures and functions until the entire genome is assigned. This ambitious project would naturally require a very large amount of work but involves no conceptual difficulties. In the words of Morowitz (177), "completely understanding the operations of a procaryotic cell, that is, a mycoplasma, is a visualizable concept, one that is within the range of the possible." If achieved, it would mean that the "logic of life" is finite, relatively simple, and subject to full exploration; a conclusion of far-reaching implications on our perception of the meaning of life.

Base Composition

The generalization that the mycoplasma genome is poor in G+C is based on data available for almost all of the established species (139, 232), and determination of the DNA G+C content has been included among the obligatory tests required for definition of new species (268). Determination of base composition of hydrolyzed DNA by high-pressure liquid chromatography has been recently added as a direct means for determining the G+C content of mycoplasmal DNAs. The values obtained by high-pressure liquid chromatography were usually in good agreement with

those obtained by the indirect methods based on thermal denaturation and buoyant density (71, 112, 116, 213). High-pressure liquid chromatography also has the advantage of providing data on modified bases (213).

The mol% G+C values are effective tools in the classification of mollicutes and of procaryotes in general (127). Thus, the finding of a difference in the G+C content between bovine ureaplasmas (28.7 to 30.2 mol%) and the human *Ureaplasma urealyticum* strains (26.9 to 28.0 mol%) served as an important indicator for the establishment of a new species, *Ureaplasma diversum*, for the bovine strains (120). A difference in the G+C content larger than 1.5 to 2.0 mol% between DNAs of two bacteria is considered sufficient to rule out their inclusion in the same species (127). The easily obtained G+C data have been found, therefore, to be extremely useful as directory means in the grouping and classification of the numerous spiroplasmas isolated recently (23, 286). It should be stressed, however, that identical or very close G+C values among microorganisms do not necessarily mean that they are genetically related, as in many of these cases the DNA nucleotide sequence, which determines genetic relatedness, differs significantly.

With the exception of *Mycoplasma pneumoniae* and some anaeroplasmas, the G+C values of all the mollicutes tested so far range between 24 and 35 mol%. Many mycoplasmas, particularly those of bovine and caprine origin, exhibit values as low as 24 mol% (39), values which are even slightly lower than the theoretical minimum of 26 mol% G+C required to code for proteins with a normal amino acid composition (73, 245). *M. capricolum* appears to have overcome the constraint imposed by the extremely high A+T content of its genome by preferential use of A- and U-rich codons, in addition to having intergenic spacers extremely rich in A+T, and rRNAs with the highest A+U content reported for procaryotes (190). Among other procaryotes such low values can be found in certain clostridia, a property used to support the notion that mollicutes are descendants of certain clostridia (see Phylogeny section).

Methylated bases. Bases modified by methylation occur at a low frequency in DNA. This modification is carried out by specific methyl transferases (DNA methylases) that transfer the chemically active methyl group from *S*-adenosylmethionine to either carbon 5 of cytosine residues (yielding 5-methylcytosine [m^5Cyt]) or to the exocyclic amino group attached to carbon 6 of adenosine residues (yielding 6-methyladenine [m^6Ade]) of the DNA chain (212, 214). In the DNA of some procaryotes a small fraction of cytosine residues is methylated, while in others only adenine residues are methylated. Some procaryotes contain both methylated bases. Eucaryotic DNA, in general, is methylated exclusively at cytosine residues (212).

Methylated bases in mycoplasmal DNA were first detected by Razin and Razin (213) by high-pressure liquid chromatography of hydrolyzed DNA. The DNAs of the five *Mycoplasma* and *Acholeplasma* species tested contained m^6Ade, while one species, *Mycoplasma hyorhinis*, contained in addition m^5Cyt (213). Recent extension of this study to spiroplasmas (202) revealed that four out of five of the spiroplasma strains examined differ from *E. coli* and from the previously tested *Mycoplasma* and *Acholeplasma* species in having m^5Cyt as the only methylated base in their DNA.

Whether or not methylation patterns can serve as genetic markers to characterize and differentiate *Mollicutes* species cannot be decided on the basis of available data. It appears that the type of base methylated, whether it is cytosine, adenine, or both, may be of relevance in this respect. However, Dybvig et al. (71) detected mostly m^5Cyt in the DNA of the K2 strain of *Acholeplasma laidlawii* and failed to detect any methylated bases in the JA1 strain, whereas in the *A. laidlawii* strains oral and JA1 examined by us (116, 202, 213) only m^6Ade could be detected. Clearly, *A. laidlawii* strains may differ in methylation patterns, but the discrepancy in data obtained for the JA1 strain should be resolved by comparing the strains studied by the two laboratories. It also appears that the extent of methylation represents an even more variable parameter than the type of base methylated, probably depending on the age of culture (202).

The methylation sequence specificity probably constitutes a more meaningful criterion for differentiating mycoplasmas, as it must reflect the nucleotide sequence recognized by the DNA methylase(s) of the organism. Two approaches can be used for determining sequence specificity of the bases methylated. The first one utilizes pairs of restriction enzymes (isoschizomers) that recognize the same site, but while one of the pair cleaves the DNA when cytosine or adenine is methylated, the other does not act when cytosine or adenine is methylated. Thus, *Hpa*II and *Msp*I are isoschizomers recognizing the CCGG sequence. However, *Hpa*II will not cleave the DNA when the cytosine residue adjacent to guanine is methylated, while *Msp*I will act when cytosine is methylated. The resistance of *Spiroplasma* sp. strain MQ-1 DNA to cleavage by *Hpa*II but not by *Msp*I indicated that most, if not all, of the cytosine residues adjacent to guanine in the CCGG sites on the genome of this spiroplasma are methylated (202). Likewise, the finding by Chan and Ross (32) that *Mycoplasma hyopneumoniae* DNA resists digestion by *Mbo*I but is susceptible to cleavage by its isoschizomer, *Dpn*I, indicated the methylation of adenine residues in the GATC sites on the chromosome, a feature differentiating this mycoplasma from the closely related species *Mycoplasma flocculare*. On the other hand, the sequence GATC in the DNA of *A. laidlawii* strain K2 appears to be methylated at the cytosine residues, as evidenced by the sensitivity of this DNA to digestion by *Mbo*I but not by its isoschizomer *Sau*3A (71). The second approach, based on nearest-neighbor analysis, has been used to determine the state of cytosine methylation in the dinucleotide sequences CpG, CpC, CpT, and CpA (101). By this method, m^5Cyt was found on the 5′ side of each of the four major bases in DNA of *S. floricola* and *Spiroplasma* sp. PPS-1, whereas in *S. apis* B31, DNA cytosine was methylated only when its neighboring base was adenine or thymine. In *Spiroplasma* sp. MQ-1, essentially 100% of the methylated cytosine was in CpG sequences, corroborating the data obtained with the restriction enzymes isoschizomers (202).

Little has been done to characterize DNA methylases in mollicutes. Preliminary experiments with a partially purified extract of *Spiroplasma* sp. strain MQ-1 showed it to methylate cytosine of double-stranded DNA of bacteriophage M13 in vitro only at the dinucleotide sequence CpG, resembling the specificity of the methylase in vivo (202). The exclusive methylation of CpG sequences is characteristic of eucaryotic methylases. Procaryotic DNAs are methylated at recognition sites containing at least four nucleotides. However, the MQ-1 methylase activity differed from known eucaryotic methylases by showing high activity on nonmethylated DNA duplexes, low activity with hemimethylated DNA duplexes, and no activity on single-stranded DNA (202). It should also be mentioned in this context that in eucaryotes only part of the CpG sites of their chromosomal DNA are methylated, while in the spiroplasma MQ-1 DNA all CpG sites are

methylated. The question of how much weight should be put on this unique observation when applied to phylogeny cannot be answered at the present. The marked genetic and phenotypic diversity of the *Mollicutes*, reflected also in their methylation patterns, has been attributed to a state of rapid evolution (see Phylogeny section). Accordingly, the presence of a CpG methylase in a member of *Mollicutes* may be another of the unexpected or bizarre properties of these procaryotes and may have little weight in consideration of their phylogenetic status.

The finding that essentially 100% of the CpG sequences in *Spiroplasma* sp. strain MQ-1 DNA are methylated was used to assess the frequency of the doublet CpG in this DNA (202). Calculations based on the expected random frequency of the CpG doublet, taking into account the G+C content of the DNA, yield a value of 2.25%. However, calculations based on the amount of m^5Cyt in *Spiroplasma* sp. strain MQ-1 DNA yielded a value of 0.45% for CpG sequences, indicating a considerable underrepresentation of this sequence in the spiroplasmal DNA. This supports the conclusions of Russel et al. (249) pointing to the underrepresentation of the CpG sequences in the DNAs of a variety of bacteria, including a few animal mycoplasmas. Moreover, underrepresentation of CpG sequences is a feature common to vertebrate DNA (19).

The biological significance of DNA methylation is still obscure. Many procaryotic-specific methylations have been shown to play a role in the restriction and modification phenomenon (71, 166), while other sequence-specific methylations such as the adenine methylation at GATC sites in *E. coli* have been shown to be associated with postreplicative mismatch repair. However, the biological significance of most DNA modifications and the evolution of the extensive diversity of DNA methylation are essentially unknown. It has been recently suggested that CpG methylation is a late development in the evolution of eucaryotic organisms aimed to modulate differential gene expressions (214). As the procaryotic *Spiroplasma* sp. strain MQ-1 does not differentiate, this may suggest that CpG methylation has other biological roles. In vitro methylation of CpG sequences has been shown to silence a variety of eucaryotic genes when introduced into cells after methylation (214). As gene inactivation by CpG methylation seems not to occur in the spiroplasma, it appears that CpG methylation, by itself, does not in general impose a physical stress on DNA sufficient to prevent its transcription. The CpG methylase of *Spiroplasma* sp. strain MQ-1 may, therefore, serve as a useful system to study the biological role of CpG methylation (202).

Base Sequences

Deoxyribonucleic acid-deoxyribonucleic acid hybridization. Identity of an organism is inherent in the sequence of the nucleotide bases in its genome, and therefore the proportion of nucleotide base sequences held in common by two organisms indicates the extent of their genetic relatedness. Since direct comparison of base sequences of procaryotic genomes is still impractical, estimation of the proportion of common base sequences has been made indirectly by a variety of DNA-DNA or DNA-RNA hybridization techniques. The early application of these techniques to mycoplasmas about 20 years ago was instrumental in putting an end to the heated controversy centered around the wrong notion that mycoplasmas are L-phase variants (L-forms) of present-day eubacteria (for a review, reference 216). Furthermore, these early hybridization data have also indicated the wide genetic heterogeneity of the mycoplasmas, reflecting the breadth of a large biological group. They have also revealed marked genetic heterogeneity within some established species, notably *Mycoplasma hominis* (216).

The relative scarcity of morphological and biochemical properties useful in mycoplasma classification has given extra weight to serological distinctions (268). However, recognition of the fact that reliance on serological properties suffers from the same limitations as dependence on other phenotypic properties has been responsible for the much wider use of molecular genetic techniques in classification of *Mollicutes*. The considerable weight given nowadays to DNA homology data requires some comment on the relative efficiency of the various techniques employed. Hydroxyapatite chromatography appears to be the method of choice for separating hybridized heteroduplexes from single-stranded DNA, as it does not suffer from the problems that plague filter-binding assays, such as leaching of immobilized DNA from the filter (265). Utilization of S1 nuclease to specifically digest unhybridized single-stranded DNA is liable to yield lower (by 15 to 20%) homology values (127), possibly explaining the relatively low homology values (32 to 37%) obtained by this technique for *Spiroplasma citri* and the corn-stunt spiroplasma (145) compared with 50 to 68% homology obtained by the hydroxyapatite and filter procedures (128, 147, 210). Nevertheless, Christiansen et al. (37), using the filter technique, also reported 30% homology between *S. citri* and the corn-stunt spiroplasma. Clearly, if DNA homology values are to be included as important criteria for determination of genetic relatedness and establishment of new species, standardization of procedures is essential. A step toward this aim was recently taken by publishing recommended procedures in *Methods in Mycoplasmology* (232). One way to support homology data is to determine thermal elution midpoints of the double-stranded DNA fractions (both homo- and heteroduplexes) eluted off the hydroxyapatite columns. These values provide a measure of the percentage of mismatched base pairs. In general, the degree of mismatching in the heteroduplex correlates directly with percentage homology, i.e., the higher the hybridization values, the more similar are the thermal elution midpoint values (265).

Taken as a whole, the rather extensive DNA homology data reported for mollicutes support the present classification of this group based mainly on morphological, biochemical, and serological characteristics. Thus, the DNA of 18 *Mycoplasma* and 3 *Acholeplasma* species tested by Sugino et al. (271) displayed a low degree of homology of the order of 3 to 5% of the genome, although a few species in each genus showed up to 15% homology. Similarly, eight established *Acholeplasma* species showed very little genetic relatedness in DNA hybridization tests apart from *Acholeplasma granularum*, which exhibited 22 to 23% homology with *A. laidlawii* (265). The real problem lies with certain species or clusters of related strains yielding hybridization data in the range of 50 to 80% homology within the species. Johnson (127) considers 60 to 70% homology as a transitional point between genetic events that may be largely cistron rearranging in nature and genetic events in which there are also many changes in base sequences. Accordingly, strains belonging to the same species should show at least 70% homology, while 60 to 70% homology justifies their separation into subspecies. Strains showing 20 to 60% homology can be included in separate, although closely related, species (127). It must be emphasized that these criteria are to some extent arbitrary, and the decision whether or not to

classify genotypically and phenotypically related strains into different species or into subspecies of the same species is a most difficult one, in light of the ill-defined species concept in procaryotes. Previous mistakes, notably those made in the classification of salmonellae, have taught us not to rush in giving new names. Thus, despite the finding that the known *U. urealyticum* serotypes form two distinct clusters distinguished by electrophoretic patterns of cell proteins (121, 186, 272) by cleavage patterns of their DNA by restriction endonucleases (229), and by exhibiting 40 to 60% DNA homology between the clusters (38), the ICSB Subcommittee on the Taxonomy of *Mollicutes* (270) considered it to be unwise to classify, at this stage, the two clusters as two separate species, or even subspecies, although this move could have been justified by the criteria of Johnson (127). Obviously, if future research will associate pathogenicity or some other important property with only one of the clusters, then a second species name might be useful (270).

Similar taxonomic and nomenclature problems are presented by other genetically heterogenous species, such as *M. hominis*, in which 10 strains exhibited a range of 52 to 100% DNA homology (15), and the *Mycoplasma mycoides* and *M. capricolum* species characterized by each consisting of a cluster of genetically heterogeneous, although related, strains (8, 39). *A. laidlawii* and *Acholeplasma axanthum* also show considerable intraspecies genetic heterogeneity (265). The variation among strains within these two established species ranged from 48 to 100% DNA homology, theoretically justifying the division of each of these species into several different species or subspecies. Another most difficult question to answer is whether the DNA hybridization data justify by themselves the separation into distinct species of the corn-stunt spiroplasma and other spiroplasmas of serogroup I related to *S. citri*, such as the honeybee spiroplasma BC-3 (22, 23, 25). In this last example, the different ecology and pathogenicity of the strains involved would seem to favor their naming as different species.

It appears that we have to live with the fact that some of the currently established species in *Mollicutes* are made up of clusters of strains exhibiting various degrees of genetic relatedness. This is not surprising in light of our rather artificial and empirical system of bacterial classification and in light of pronounced genetic drifts or genetic instability that could be brought about by plasmid or viral DNA integration into the genome of mollicutes (64, 69, 180, 181; I. Nur, G. Glasen, and S. Razin, submitted for publication). It could be expected that genetic drifts take place continuously under natural conditions and no doubt occur in field strains adapted to grow in vitro and during continuous subcultivation. It could also be argued that genetic heterogeneity among strains of the same species is associated with their recovery from different habitats. Adaptation of an organism to a new host would pressure it to genetic changes to survive. If correct, this hypothesis could explain the marked genetic heterogeneity of the *A. axanthum* and *A. laidlawii* strains isolated from a diversity of hosts, and the remarkable genetic homogeneity exhibited by mycoplasmas such as *M. pneumoniae*, characterized by strict host and tissue specificity (33, 233, 265).

Deoxyribonucleic acid cleavage patterns. Availability of restriction endonucleases recognizing specific nucleotide sequences in DNA molecules has led to development of a new approach for assessing genetic relatedness among mollicutes. The cleavage patterns produced by electrophoresis of the digestion products of mycoplasmal DNAs by restriction enzymes provide valuable information on the type and number of specific nucleotide sequences, as well as on methylated bases (see Methylated bases section). The use of *Eco*RI cleavage patterns as a taxonomic aid was first proposed by Bove and Saillard (24). They encountered some difficulty in the application of this method to spiroplasmas. The inconsistent presence of extrachromosomal DNA (plasmids, viruses) in these mollicutes introduced variability in the cleavage patterns, hampering their use in strain identification (25). The successful application of the restriction enzyme approach to detection and identification of mycoplasmas infecting cell cultures (50–52, 238) served as a boost for critical evaluation of the potential of restriction endonucleases as tools in determining genetic relatedness among strains of various *Mycoplasma*, *Ureaplasma*, and *Acholeplasma* species (33, 112, 225, 229, 233). Restriction endonucleases having six-nucleotide recognition sequences are preferable to those having four-nucleotide recognition sites, since they cleave the mycoplasma genome into fewer fragments. Moreover, the small size of the mycoplasma genome and its generally low G+C content proved to be of great advantage, as restriction endonucleases with recognition sites rich in G+C cleave the mycoplasma genome at relatively few sites. As a result, the electrophoretic patterns of cleavage products exhibit a limited number of bands and are easier to compare (225, 229, 233).

Strains of the established pathogens *M. pneumoniae* and *M. gallisepticum* and of the newly discovered *Mycoplasma genitalium* showed remarkably similar species-specific cleavage patterns, indicating the genetic homogeneity of these three species (33, 233). The marked genetic homogeneity of *M. pneumoniae* strains has also been supported by DNA hybridization data and by electrophoretic patterns of cell proteins (33). On the other hand, strains of *A. axanthum* and *M. hominis* that showed considerable intraspecies genetic heterogeneity in DNA hybridization tests (15, 265) also differed markedly in their cleavage patterns (15, 233). Division of the *U. urealyticum* serotypes into two genotypically different clusters, according to DNA hybridization and electrophoretic patterns of cell proteins, also gained strong support from DNA cleavage patterns (229).

The major advantage of the DNA cleavage approach over the more cumbersome DNA-DNA hybridization stems from the fact that it requires only a few micrograms of unlabeled DNA, and the results can be read and compared visually very easily. The cleavage technique serves as an excellent means for testing clonality and identity of strains. Thus, we use this technique routinely for checking identity and purity of our *M. genitalium* clones. Replacement by mistake of a *M. genitalium* culture by *M. pneumoniae* was easily detected in this way (Fig. 1). It should be recalled that it is difficult to distinguish between these two mycoplasmas by growth characteristics and by serology (148). Similarly, chromosomal cleavage patterns clearly distinguished *M. hyopneumoniae* from *M. flocculare*, two organisms hard to differentiate by conventional identification techniques (32).

The deficiencies of the restriction enzyme approach derive from the fact that the tested DNA must be pure and undegraded, a feat hard to achieve with some mycoplasmas containing particularly potent nucleases (233). In addition, although cleavage patterns are adequate for determining identity, close similarity, or nonidentity of strains, it is difficult to determine degree of relatedness on the basis of the patterns, as cleavage data are not expressed by numbers, unlike hybridization data (225). As was mentioned above, the presence of extrachromosomal DNA may complicate

reading of results. Nevertheless, this deficiency can be turned into an advantage, as the presence of one or two heavy bands in a cleavage pattern may indicate the presence of a plasmid or a virus, as very frequently their DNA has only one or two restriction sites for the tested enzyme (Nur et al., submitted). However, in the case of more restriction sites or upon integration of considerable amounts of plasmid DNA into the chromosome cleavage patterns can be expected to differ considerably from those of the uninfected strain. It should also be mentioned here that restriction endonucleases sensitive to the presence of a methylated base in their recognition site may not digest the DNA. For example, the *A. laidlawii* JA1 DNA was digested by *Sau*3A, which recognizes the sequence GATC, while the closely related *A. laidlawii* K2 DNA was not cleaved by this enzyme, because the K2 cells have a methylase specific for cytosine at the sequence GATC (71).

Electrophoretic patterns of cell proteins. Electrophoretic analysis of cell proteins, the products of DNA translation, provides an alternative, if less complete, way than DNA hybridization for comparing the genomes of mycoplasmas. This poor man approach to determining genetic relatedness was first introduced by Razin and Rottem in 1967 (231), using one-dimensional acidic PAGE and cylindrical gels. The later introduction of sodium dodecyl sulfate (sodium dodecyl sulfate-PAGE), slab gels, labeling of cell proteins by growth with radioactive amino acids, and fluorography improved resolution considerably, enabling visualization of over 60 different protein bands in each electropherogram (49, 185, 187). A much greater degree of resolution, approaching the separation of individual cell proteins, has been achieved by application of 2D-PAGE to mycoplasmas by Rodwell and Rodwell (241) and by Mouches et al. (187). The method is particularly well suited for mycoplasmas, since due to their minute genome, the protein fingerprints are relatively less complex and more readily interpretable than those of other microorganisms. Over 300 protein spots can be distinguished by prolonged fluorography of gels of *Mycoplasma* species having a 500-MDa genome (129, 240, 241). This represents more than half of the expected coding capacity of the genome (see Genome Size section). Too long an exposure of gels to the film affects resolution, since minor spots lying close to major ones are engulfed before they can be detected. For easing comparison of protein profiles, exposure times yielding about 150 to 190 spots are recommended by Rodwell (240). In keeping with their larger genome (1,000 MDa), the 2D-polyacrylamide gels of acholeplasmas are more complex than those of *Mycoplasma* species; they exhibit about 300 spots under exposure periods yielding only about 150 spots for *Mycoplasma* species (240).

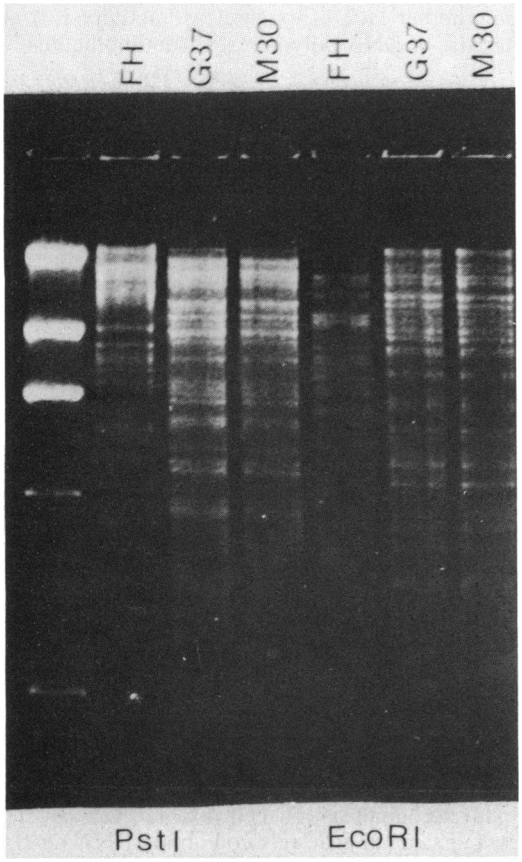

FIG. 1. Cleavage patterns of the DNA of *M. genitalium* M30 and G37 and of *M. pneumoniae* FH by the restriction endonucleases *Eco*RI and *Pst*I. The identity of the cleavage patterns of the two *M. genitalium* strains and their dissimilarity to the pattern of the *M. pneumoniae* strain can be seen (D. Yogev and S. Razin, unpublished data).

Generally, conclusions concerning the taxonomic status of species arrived at by 2D-PAGE corresponded very well with those obtained by the DNA hybridization and restriction enzyme techniques. For example, the division of *U. urealyticum* strains into two genetically different clusters, as indicated by DNA hybridization (38) and by DNA cleavage patterns (229), was observed also by 2D-PAGE of cell proteins (186). The genetic relatedness of *M. capricolum* to strains of Leach group 7 and F38 group, detected by DNA hybridization (39), was confirmed by 2D-PAGE (8, 240). 2D-PAGE also proved to be most useful, in addition to DNA hybridization, in determining genetic relatedness and taxonomic status of spiroplasmas (184, 185, 187).

Some comments should be made on the reading and evaluation of the 2D-PAGE data. One may distinguish three classes of proteins on comparing protein maps of two tested strains: (i) comigrating proteins, apparently identical; (ii) homologous proteins which have slightly different migrational properties from one isolate to the other; and (iii) proteins which are present in one strain and absent in the other, apparently different proteins. Thus, comparison of the protein maps of the genetically related *S. citri* and corn-stunt spiroplasma revealed 19% comigrating proteins and 33% homologous proteins, whereas maps of two *S. citri* strains showed over 90% comigrating proteins (185). Yet, Rodwell (240) concludes on the basis of his extensive experience with the 2D-PAGE technique (91 strains of 19 species tested) that considerable variations in protein patterns can be found among strains of the same species. The percentage of matching (comigrating) spots (percentage of congruence) ranged in his studies from 42 to 100%, but many proteins were strongly conserved in all strains of a given species. According to Rodwell (240), in interpreting the protein patterns of different strains we assume that congruent spots are composed of isofunctional proteins of identical, or nearly identical, amino acid sequence. Isofunctional proteins with extensive amino acid sequence homology may be resolved by 2D-PAGE and classified as distinct and unrelated proteins, as isoelectric focusing is potentially capable of resolving proteins differing by a single charge. Therefore, strains which are indistinguishable by DNA hybridization may reveal significant differences in protein patterns. It should

also be recalled that the number of spots usually represents only about one-quarter to one-third of the expected coding capacity of the genome and that not all substitutes or deletions of amino acids would result in a significant change in mobility of a protein. Nevertheless, despite all the above limitations and reservations, 2D-PAGE enables the examination of 150 to 190 characteristics at one time. It is difficult to envisage any other method that could have greater specificity with the possible exception of pyrolysis mass spectrometry (240).

GENOME REPLICATION

Site of Replication

As discussed in my previous review (218), in many mycoplasmas cytoplasmic division lags behind genome replication, resulting in the formation of multinucleate filaments. The filaments transform later into chains of cocci by constriction around each of the chromosomes, finally disintegrating into single cocci. Similarly, the helical filaments of spiroplasmas divide by constriction to produce elementary helices (81, 82).

Search for the DNA replication site in *M. gallisepticum* led Maniloff and Quinlan (170) to suggest that it is associated with the peculiar bleb of this organism. According to their hypothesis a new bleb, formed at the site of replication, moves with the attached new daughter chromosome to the opposite pole of the cell before cell division to ensure chromosome segregation (162). Movement of the bleb could be effected by a contractile system, the existence of which has been proposed by several authors to explain shape, filament helicity, cell division, and motility in mollicutes (for references, see references 195 and 219). Maniloff (162) found that cytochalasin B, a drug affecting actin, increases the number of *M. gallisepticum* cells with two blebs and shuts off DNA synthesis two generations after its addition. Labeled cytochalasin B was found to bind to the blebs and to the cell membrane and, according to Maniloff, acts by inhibiting an actin-like protein. In short, Maniloff proposes that the bleb structure be regarded as the procaryotic analog of the eucaryotic centriole. Although this analogy may hold for *M. gallisepticum*, it clearly does not apply to any mycoplasma lacking terminal structures, and these are the majority of *Mollicutes*. In fact, Maniloff (162) points out that cytochalasin B inhibits growth of *M. gallisepticum* but not of several other mycoplasmas tested by him.

Deoxyribonucleic Acid Polymerases

The only characterized components of the DNA replication complex in mollicutes are several DNA polymerases. Although information is limited to a few organisms (*Mycoplasma orale*, *M. hyorhinis*, *S. citri*, and several other spiroplasmas), some interesting and unique properties of the enzymes came into light. The early work of Mills et al. (173) on properties of the *M. orale* and *M. hyorhinis* DNA polymerases has been discussed in previous reviews (218, 261). Both mycoplasmas appear to contain a similar single DNA polymerase, in contrast to the finding of three DNA polymerases in *E. coli* and other procaryotes. More recently, Boxer and Korn (27) purified the *M. orale* polymerase to homogeneity. It consisted of a single protein of 116 kDa. The purified enzyme resembled the partially purified preparation of Mills et al. (173) in being completely devoid of exo- and endo-deoxyribonuclease (DNase) activities. Otherwise, the purified enzyme possessed the catalytic properties exhibited by the characteristic eubacterial DNA polymerases. Since the fidelity of the *M. orale* DNA polymerases resembles that of *E. coli* polymerase I, the nature of the proofreading function in the mycoplasma is unclear in the absence of the 3′→5′ exonuclease activity. All the eubacterial DNA polymerases studied, with the possible exception of *B. subtilis* DNA polymerase II (261) contain 3′→5′ exonuclease activity.

Can the finding of a single DNA polymerase lacking exonucleolytic activities provide a biochemical marker indicative of substantial divergence between mollicutes and eubacteria, as proposed by Maniloff (164), or does it just represent another of the bizarre properties of *Mollicutes* (291)? The observations of Charron et al. (34, 35) cast doubts about the generality of the above properties by showing the presence of three DNA polymerases in *S. citri*, in the related honeybee *Spiroplasma* sp. strain BC-3, and in the flower spiroplasma *S. floricola* (BNR1). The question of whether the spiroplasmal DNA polymerases lack the 3′→5′ exonuclease activity as the *M. orale* and *M. hyorhinis* DNA polymerases do has not been definitely answered. Charron et al. (34) mention that the two *S. citri* DNA polymerases identified at that time appeared to lack this property. No mention of this question can be found in their later report (35). However, more recently, Bove (23) cites unpublished work of A. Charron suggesting that an unidentified acholeplasma DNA polymerase possesses exonuclease activities. Clearly, more work has to be done to answer the question whether lack of exonuclease activity is a general characteristic of DNA polymerases from mollicutes.

Nucleases

The wide occurrence in mollicutes of nucleases hydrolyzing DNA and RNA was established long ago (206, 230, 242). Interest in nucleolytic activities of mollicutes stems from their possible role in mycoplasma nutrition, by providing assimilable nucleic acid precursors from polymeric DNA and RNA (216, 230), their possible role as pathogenicity factors damaging host-cell nucleic acids (for references, see reference 206) and their interference with isolation of undegraded mycoplasmal DNA adequate for molecular genetic studies (233). Obviously, the endogenous nucleases may have additional functions, such as in genetic recombination, DNA repair, restriction of foreign DNA, and transport of transforming DNA. Recognition of these possible roles prompted several recent studies aimed at the characterization of the mycoplasmal DNases, particularly those of acholeplasmas (206, 242). Extracts of several *Acholeplasma* and *Mycoplasma* species were tested for DNase and endonuclease activities. All extracts revealed both activities, as they hydrolyzed both *E. coli* DNA and superhelical covalently closed circular PM2 form I DNA (206). More detailed analysis of *A. laidlawii* fractions obtained by sucrose gradient centrifugation indicated the presence of several endonuclease activities with different substrate specificities, which unfortunately were not characterized further. The great variety of DNases in acholeplasmas has also been verified by electrophoretic analysis of cell extracts of seven *Acholeplasma* species in agar gels containing DNA (242). Each species yielded a species-specific pattern of DNase-containing bands.

The only characterized mycoplasmal endonuclease is the site-specific endonuclease named *Sci*NI partially purified from *S. citri* (267). This enzyme is an isoschizomer of *Hha*I, recognizing the sequence 5′-GCGC-3′. It cleaves between

the first G and C, while *Hha*I cleaves between the second G and C. The *Sci*NI-generated end possesses a two-base 5′ extension, which could act as a template for the 5′→3′ polymerase activity of the T4 polymerase (267). As expected, the *S. citri* extract also contained a nonspecific DNase which degraded DNA without producing distinct bands in agarose gel electrophoresis. Neither *Sci*NI nor *Hha*I cleaved the *S. citri* DNA, despite the fact that the sequence 5′-GCGC-3′ should occur approximately 300 to 400 times in the genome of this organism, suggesting that the *Sci*NI sites are protected in *S. citri* DNA by base modification. Although Stephens (267) observed classical restriction and modification of viruses in some *S. citri* strains, he failed to establish a correlation between the *Sci*NI activity with restriction and modification systems, leading him to propose that in these strains other site-specific endonucleases are operative. Obviously, investment of more work in the characterization of site-specific endonucleases in mollicutes will be most rewarding.

As mentioned above, the rather intensive endogenous DNase activity in mollicutes, particularly in acholeplasmas, hampers isolation of undegraded DNA from the organisms. The fact that the enzymes require Mg^{2+} for activity (206, 230, 267) promotes the use of ethylenediaminetetraacetic acid to shut off nucleolytic activities during isolation of cellular DNA. However, in the case of *A. laidlawii* and possibly other mollicutes with very high endogenous DNase activities, this does not always work (233). Pollack and Hoffman (206) recommend the addition of 1 to 5 mM Zn^{2+} in case ethylenediaminetetraacetic acid does not suffice to stop endogenous DNase activity. Interestingly, these authors (Pollack and Hoffman, personal communication) have found that 0.5 M NaCl completely inhibits the DNase activity of *M. pneumoniae*, *M. pulmonis*, and *M. arthritidis*, but only partially inhibits that of *A. laidlawii*.

IRRADIATION DAMAGE AND MUTAGENESIS

Ultraviolet Irradiation Damage and Repair

Mollicutes are very sensitive to ultraviolet (UV), X-, and gamma-ray irradiations, probably more than other procaryotes when inactivation values are normalized per unit of DNA (36, 75, 140, 169). The survival curves of mycoplasmas irradiated by UV usually exhibit a shoulder near the origin, followed by exponential inactivation or killing (9, 140, 169). A curve of this shape, characteristic of multiple-hit kinetics, may be due to cell clumping or polyploidy, both common features of mycoplasmas in culture. The shoulder may also be indicative of the existence of DNA repair systems (85, 140, 204).

Both photoreactivation and dark (excision) repair mechanisms have been shown to operate in *A. laidlawii* (59) and in *S. citri* (J. Labarere, personal communication). Acriflavine and caffeine, agents known to inhibit excision repair by binding to the DNA, abolished dark repair in UV-treated *A. laidlawii* (85) and in several human mycoplasmas (9). Nevertheless, it appears that some mycoplasmas are deficient in DNA repair systems. Thus, Aoki et al. (9) could not detect photoreactivation in *Mycoplasma buccale*. The extreme case, however, is represented by *M. gallisepticum*, in which Ghosh et al. (84) failed to find either dark repair or photoreactivation. This is an unexpected and surprising finding, as it is difficult to envision an organism lacking systems essential for maintaining the high fidelity of genome replication. *M. gallisepticum*, being an avian respiratory pathogen, is likely to be exposed to UV irradiation during its transmission from one host to another. Maniloff's suggestion (162) that, due to the lack of DNA repair mechanisms, *M. gallisepticum* may accumulate base changes far more frequently than other procaryotes, has been cited in support of the notion of rapid evolution in mollicutes (290, 291). However, experimental support for higher mutation rates in *M. gallisepticum* is not available. On the contrary, *M. gallisepticum* appears to be one of the genotypically homogeneous species, as judged by the finding of only slight variations among its strains in DNA cleavage patterns (233), cell protein composition, and antigenic structure (215, 237). Furthermore, the recent finding (204) of lack of dark repair in *A. laidlawii* K2 indicates that there is no simple relationship between DNA repair capability and mycoplasma evolution (J. Maniloff, personal communication).

Mutagenesis

N-Methyl-*N*′-nitro-*N*-nitrosoguanidine (nitrosoguanidine) has been successfully used as a mutagenic agent in the search for temperature-sensitive mutants of *M. pneumoniae* (262), *M. gallisepticum* (141), immune-lysis resistant mutants of *A. laidlawii* (47), and hemadsorption-negative (HA^-) mutants of *M. pneumoniae* (74, 110). Increases in the frequency of mutation in mollicutes by nitrosoguanidine were of the same order of magnitude as noticed with other procaryotes. An interesting observation concerns the hemadsorption-negative mutants of *M. pneumoniae*. Krause et al. (138), while extending the work of Hansen et al. (110), found that the rate of spontaneous loss of the hemadsorption property from the wild-type *M. pneumoniae* strain was very high, eliminating the need for a mutagenic agent. Isolating spontaneous mutants has the advantage of minimizing the possibility of induction by a mutagen of secondary mutations unrelated to adherence (see also reference 262). Surprisingly, the rate of spontaneous appearance of nonhemadsorbing clones was so high (7×10^{-3}) that it questioned their definition as mutants. Krause et al. (138) justify the use of the term mutants on the grounds that the nonhemadsorbing clones retained the HA^- phenotype upon repeated passages. Reversion to the HA^+ phenotype occurred only at a low frequency, so that a selective procedure based on repeated isolation of erythrocyte-associated mycoplasmas had to be applied (137). The finding that the spontaneous and nitrosoguanidine-induced HA^- clones showed similar protein profiles and biological properties (138) can also be added in support of the identification of the HA^- clones as mutants. Obviously, the observation of a high spontaneous mutation rate in a mycoplasma can be brought up in support of the Woese hypothesis (291). However, Feldner and Bredt (74) reported that many of their nitrosoguanidine-induced HA^- *M. pneumoniae* "mutants" reverted spontaneously to HA^+ during cloning, again casting doubts about their being true mutants.

Recently, Labarere and Barroso (140) used UV irradiation to induce *S. citri* mutants resistant to arsenic acid and to xylitol. Irradiation increased the spontaneous mutation frequency of resistance to xylitol by 5×10^2 and to arsenic acid by 1×10^2. Thus, UV irradiation appears to be effective in obtaining a large number and variety of mutants. Moreover, the use of UV irradiation is specially suited for obtaining only chromosomal mutations in the plasmid- and virus-infected *S. citri*, as this irradiation is supposed to cure the organisms of extrachromosomal elements (140).

TRANSFORMATION, TRANSFECTION, AND RECOMBINATION

Transformation

The lack of a cell wall in mollicutes would seem to favor genetic exchange by transformation. Yet early experiments by Folsome (75) to induce resistance to streptomycin in *A. laidlawii* by incubating streptomycin-sensitive cells with DNA of a streptomycin-resistant *A. laidlawii* strain failed. The reasons for this failure remain unclear, as the organisms were shown to bind the high-molecular-weight DNA in a temperature-dependent manner in amounts reaching 0.1 to 0.2 of the genomic DNA content of the organism within 30 min. The resistance of the bound DNA to exogenous DNase indicated that most of it penetrated the cells. These results and Folsome's data on UV inactivation of *A. laidlawii*, suggesting the absence of a dark-repair mechanism, led him to conclude that the exogenous DNA was not genetically integrated into the chromosome, because of the lack of one or more steps necessary for genetic integration. The subsequent demonstration of dark repair in *A. laidlawii* (59) speaks against this interpretation. The extremely potent DNase activity of *A. laidlawii* (see Nucleases section) can also be expected to hinder transformation experiments, although Folsome (75) states that this could not be the cause for his failure.

Many transformation experiments carried out in other laboratories also gave inconsistent results and consequently remained unpublished. The first report of successful transformation in mycoplasmas was published in 1979 by Furness and Cerone (80). DNA extracted from a tetracycline-resistant *M. hominis* strain transformed tetracycline-sensitive strains of *M. hominis* and *Mycoplasma salivarium* to tetracycline resistance. Further studies by this group (31, 251) have established the optimal conditions required for transformation and provided supporting evidence for definition of the phenomenon as transformation. Competence of cells was achieved by their preincubation for 30 min at 2 to 4°C in tris(hydroxymethyl)aminomethane buffer containing 100 mM $CaCl_2$. Uptake of exogenous DNA was relatively fast, as a 5-min incubation sufficed for some transformation, although maximum DNA binding and transformation occurred after 30 min. DNase treatment during incubation abolished transformation, as did adding to the reaction mixture nonspecific mycoplasmal or thymus DNA. These DNAs apparently compete with the specific DNA carrying the loci for tetracycline resistance. The optimal rate of transformation to resistance to 10 μg of tetracycline per ml was one transformant per 10^7 viable mycoplasmas. No spontaneous mutants to this drug concentration could be detected among 10^{10} organisms. Yet the maximum level of tetracycline resistance achieved by transformation (30 μg of tetracycline per ml) was lower than that of the donor strain (100 μg/ml). Finally, the failure by Saha et al. (251) to detect extrachromosomal DNA in the donor and the recipients by CsCl-ethidium bromide buoyant density centrifugation and agarose gel electrophoresis was taken in support of the thesis that tetracycline resistance was achieved by transformation and recombination of chromosomal genes rather than by transfection of a plasmid (R factor).

An unexpected explanation for tetracycline resistance in clinical isolates of *M. hominis* has recently been provided by Roberts et al. (239). Chromosomal DNA of the resistant strains was found to carry sequences homologous to the streptococcal tetracycline determinant *tetM*. No such sequences could be detected in tetracycline-sensitive *M. hominis* strains. Whether the *tetM* spread directly from streptococci to *M. hominis* or originated from a common source, as yet unidentified, are intriguing questions for which we have no answers.

Clearly, these studies should be extended to other mycoplasmas before any definite conclusions are drawn as to the availability of transformation as a general technique for studying mycoplasma genetics. A stride in this direction was recently taken by Goulay et al. (91), who reported the successful transformation of *A. laidlawii* B to neomycin resistance, simply by incubating neomycin-sensitive organisms in culture with DNA of a neomycin-resistant *A. laidlawii* B. The marked osmotic sensitivity of many mollicutes and their tendency to lose viability when suspended in buffer solutions could be expected to hinder transformation experiments. However, the inclusion of $CaCl_2$ in the transformation buffer and the low temperature employed would tend to cushion osmotic-shock effects (216). Another more general problem hampering genetic studies in mollicutes concerns the scarcity of genetic markers. It is difficult to select for the common type of biochemically deficient mutants, because defined media are available for very few mollicutes, and even these are too complex and consequently of little value in selection of this type of mutant. Hence, differences in susceptibility to chemotherapeutic or toxic agents are the genetic markers most commonly used in mycoplasma genetic studies.

Transfection

The first to show transfection in mollicutes were Liss and Maniloff (155). DNA isolated from two strains of the MVL1 group (see Mycoplasma Viruses section) was infectious on *A. laidlawii* JA1 lawns. Infectivity was destroyed by DNase but was resistant to ribonuclease, pronase, and virus-specific antiserum. The host cells were competent for transfection for only a relatively short time, reaching maximal competence at the late-logarithmic phase of growth. The addition of ethylenediaminetetraacetic acid improved competence, probably by inhibiting the endogenous DNases of the host (156). Compared with infection by intact virus, transfection exhibited a longer latent period and a smaller virus yield. Viral DNA exhibited a very low efficiency of infection; one infectious center required about 4×10^5 virus equivalents of DNA (156), but this was still within the range reported for other phage transfection systems.

More recently, DNA of the MVL2 virus (Table 2) was also shown to be able to transfect *A. laidlawii* (258). However, simple mixing of the DNA with the cells, as was done by Liss and Maniloff (155) with MVL51 DNA, did not suffice to induce transformation. Competence was achieved by the addition of polyethylene glycol (PEG 8000) to logarithmic-phase cells. The mechanism of the polyethylene glycol action is unclear. Apparently, it does not involve extensive cell fusion but rather membrane changes facilitating exogenous DNA binding and penetration. Again, as with MVL1 DNA, transfection efficiency of MVL2 DNA was very low: about one transfectant per 10^7 MVL2 DNA molecules and 10^{-4} transfectants per colony-forming unit. The possible existence of two *A. laidlawii* subpopulations with the smaller one (of the order of 10^4 cells) being the only one competent for transfection did not gain experimental support (258). It is of interest to mention here that the host-cell modification and restriction noticed with intact MVL2 virions (71) was also observed with transfecting DNA (see also Mycoplasma Viruses section).

The transfection system containing polyethylene glycol that was developed for MVL2 was also found to be effective in transfecting *A. laidlawii* with single-stranded DNA of MVL51 and its double-stranded replicative form (RF) (259). For both DNAs transfection frequencies were in the range of 10^{-8} transfectants per DNA molecule and 10^{-3} transfectants per colony-forming unit. Interestingly, both of the DNAs were able to transfect the *A. laidlawii* REP^- variant, resistant to infection by intact virions, due to a block in RF replication (see Mycoplasma Viruses section). Efforts to transfect *A. laidlawii* with MVL3 DNA by using the polyethylene glycol transfecting system have failed. Sladek and Maniloff (259) suggest that this failure may be due to the fact that the MVL3 DNA is much larger than that of MVL2 and MVL51, and is linear. Linear MVL2 DNA transfected at a much lower frequency than the native circular MVL2 molecules (258).

Another recent report on mycoplasma transfection concerns the transfection of spiroplasmas by DNA of the spiroplasma virus SPV4 (235). The single-stranded DNA of this virus successfully transfected the indicator spiroplasma strain G1 (honeybee spiroplasma, serogroup I-2) treated with 40% polyethylene glycol. It also transfected a clone of this spiroplasma made resistant to the intact virus. The efficiency of transfection with the SPV4 DNA reached 10^2 to 10^3 plaque-forming units per μg of viral DNA, resembling the values obtained with DNA of acholeplasma viruses.

Demonstration of transfection in mollicutes opens the way for its application as an effective tool for genetic exchange. We still do not have a shuttle vector capable of replication in *E. coli* as well as in a mycoplasma. Viral DNA capable of transfecting susceptible mollicutes can thus be used as a vector of genetic elements. Since SPV4 is a lytic virus, it may not be the best choice for the above purpose. DNA of the nonlytic SPV1 or of the nonlytic *A. laidlawii* viruses MVL1 and MVL2 may be a better vector.

Another approach for transfer of genetic elements has been based on liposome-encapsulated DNA, assuming that fusion of mycoplasma cells with liposomes will facilitate entry of the exogenous DNA into the cells. The experiments, carried out by Nicolau and Rottem (198), used the pBR322 plasmid of *E. coli* encapsulated in phospholipid vesicles and *M. capricolum* cells in culture. About 12 h after contact, the *M. capricolum* cells started to exhibit β-lactamase activity and tetracycline resistance, properties endowed by the plasmid DNA. This indicates that the transcription and translation machinery of the mycoplasma recognizes the pBR322 plasmid sequences. The transformation rate, as expressed by tetracycline resistance, was 10^{-6} (out of about 10^{-9} cells), well beyond the spontaneous mutation rate in these cells, but still relatively low, suggesting that fusion (if it is really the mechanism for DNA transfer) is a rare event.

Recombination Following Membrane Fusion

Information is extremely scarce as to the possibility of recombination in mollicutes, that is, transfer of genetic material through direct contact between cells. The mixing together of *S. citri* mutants resistant to xylitol and to arsenate, incubation at 32°C for 90 min, and plating on a medium containing both toxic substances yielded resistant colonies at a recombinant frequency of 5×10^{-5}, a value obtained after subtracting spontaneous mutation (140). Since polyethylene glycol promoted recombination and DNase and ribonuclease had no effect (J. Labarere, personal communication), the mechanism of DNA transfer apparently involves direct contact and possibly areas of fusion of the cell membranes. Supporting this idea is a recent report by Goulay et al. (91). The mixing together for 1 min at 30°C of neomycin-resistant and chloramphenicol-resistant *A. laidlawii* B cells in a culture medium supplemented with 40% polyethylene glycol yielded organisms resistant to both antibiotics at a recombinant frequency of 5×10^{-6}. These results, as well as those of Lam et al. (141) obtained with *M. gallisepticum*, suggest that recombinant events following membrane fusion do occur in mollicutes, perhaps at a frequency higher than that for wall-covered bacteria.

GENE EXPRESSION

Ribonucleic Acid Polymerase

The mycoplasmal DNA-dependent RNA polymerase, the key enzyme in transcription, appears to be resistant to rifampin. This antibiotic is an effective inhibitor of the RNA polymerase of *E. coli* and of other eubacteria and consequently inhibits growth of these organisms. The findings that growth of a variety of mollicutes, including acholeplasmas (56), mycoplasmas (124), ureaplasmas (61), and spiroplasmas (22, 23) is not inhibited by rifampin has indicated that the target enzyme, the RNA polymerase of mollicutes, is resistant to this drug. In fact, Das and Maniloff (56) could show that the RNA polymerase activity in a crude extract of *A. laidlawii* cells was highly resistant to rifampin. Thus, 50 μg of this antibiotic per ml only inhibited 50% of the in vitro polymerase activity. More recently, RNA polymerases of several spiroplasmas were purified to a state at which the enzymes required added DNA for activity. The concentrations of rifampin inhibiting 50% of the polymerase activity of these preparations were about 1,000-fold higher than those required for 50% inhibition of the *E. coli* enzyme (22, 23). Bove (22, 23) cites preliminary unpublished data (A. Gadeau, C. Mouches, and J. M. Bove) suggesting that the structure of the RNA polymerase of *Spiroplasma* sp. strain B88 resembles that of eubacteria in consisting of four protein subunits (130, 117, 49, and 40 kDa), but the two larger subunits were significantly smaller than the two large subunits (β and β′) of the *E. coli* enzyme.

It should be recalled that rifampin blocks transcription initiation by binding to the β subunit of the eubacterial RNA polymerase. It would be of interest, therefore, to find out whether the spiroplasmal enzyme subunit analogous to the β subunit in function binds rifampin. In any case, the finding of four major subunits in the spiroplasmal RNA polymerase, if extended to RNA polymerases of other mollicutes, places them closer to the RNA polymerases of eubacteria than to those of archaebacteria. The archaebacterial RNA polymerases are more complex than those of eubacteria and consist of more subunits, but resemble the mycoplasmal RNA polymerases in being resistant to rifampin (293).

Promoters, Terminators, and Control Mechanisms

The first information on promoters and terminators in a mycoplasmal genome has become available only very recently upon sequencing specific regions of a cloned gene cluster coding for mycoplasmal tRNAs (245). Rogers et al. (245) constructed a recombinant plasmid carrying a 7-kilobase (kb) insert of *Spiroplasma* sp. strain BC-3 DNA with a gene cluster coding for seven tRNA genes. When a 170-bp region upstream was sequenced, the first tRNA gene revealed an almost perfect consensus sequence for a Pribnow box (−10 region) of an RNA polymerase promoter

TABLE 2. Characteristics of mycoplasma viruses

Virus group	Virus designation	Host	Morphology	Composition			References[a]
				Nucleic acid	Proteins (polypeptides) (kDa)	Lipids	
Acholeplasma viruses							
A1 (L1)	MV-L1 (MVL1; MVL51)	*A. laidlawii*	Naked rod (71–90 by 12–16 nm)	Circular single-stranded DNA, 1.5 × 10^6 Da	Structural: 70, 53, 30, 19. Replicative intermediates: 14, 10	None	57, 201
A2 (L2)	MV-L2 (MVL2)	*A. laidlawii*	Enveloped irregular spheres (70–13 nm diam)	Circular double-stranded superhelical DNA, 7.8 × 10^6 Da (11.8 kbp)	Major proteins: 74, 69, 65, 14. 5–6 minor proteins	10–12% of total dry wt. of virus. Resemble host membrane lipids	100, 116, 158, 200, 204
A3 (L3)	MV-L3 (MVL3)	*A. laidlawii* (*A. oculi*; *A. modicum*?)	Polyhedron (~60-nm diam) with short tail (9 by 25 nm); collar with fibers	Linear double-stranded DNA, 26 ± 0.6 × 10^6 Da	Major proteins: 81, 68, 43. Minor proteins: 172, 73	None	83, 103
Not determined	MV-M1	*A. modicum*	Enveloped irregular spheres (105–160-nm diam)	ND[b]	ND	ND	46
Not determined	L172 (MV-Lg-pS2-L172)	*A. laidlawii*	Enveloped irregular spheres (60–80-nm diam)	Circular single-stranded DNA, 4.6 × 10^6 Da (14.0 kb)	Major proteins: 68, 53, 42, 15. Minor proteins: 71, 40, 18	ND	70
Mycoplasma viruses							
M1 (B1)	MV-Br1	*M. bovirhinis*	Polyhedron (77-nm diam) with long contractile tail (104 nm long) and base plate with tail fibers	ND	ND	Apparently none	97
M3	MV-Hr1	*M. hyorhinis*	Polyhedron (34-nm diam) with short tail (14 nm long)	DNA (not characterized)	ND	ND	99
M2	MV-Mp1	*M. pulmonis*	Polyhedron (31–33-nm diam); medium tail (19–26 by 6 nm)	DNA (not characterized)	ND	ND	44
Spiroplasma viruses							
S1 (C1)	SPV1 (SV1)	*Spiroplasma* species	Naked rod (230–280 by 10–15 nm)	Single-stranded circular DNA, 2.8 × 10^6 Da	ND	None	63
S2 (C2)	SPV2 (SV2)	*S. citri* (only microscopically observed, not propagated as yet)	Polyhedron (52–58 by 48–51 nm) with long tail (75–83 by 6–8 nm)	ND	ND	ND	43
S3 (C3)[c]	SPV3 (SV3)	*Spiroplasma* species	Polyhedron (37–44 by 35–37 nm) with short tail (13–18 by 6–8 nm)	Linear double-stranded DNA, 16 × 10^6–20 × 10^6 Da, may be circularly permuted	5–7 proteins; molecular sizes depending on strain	None	43, 63, 65, 278

Continued on following page

TABLE 2—*Continued*

Virus group	Virus designation	Host	Morphology	Composition			References[a]
				Nucleic acid	Proteins (polypeptides) (kDa)	Lipids	
S4 (C4)	SPV4 (SV4)	Bee spiroplasma (serogroup I-2)	Naked isometric sphere (27-nm diam)	Circular single-stranded DNA; 1.7 × 10^6 Da	One major protein, ~60; minor proteins probably present	ND	235, 236

[a] For general references see Maniloff et al. (168).
[b] ND, Not determined.
[c] This group probably includes the viruses designed spv-1 and spv-2 shown to infect the sex-ratio spiroplasma in *Drosophila* species (203, 288). These viruses have not been propagated and characterized as yet.

region. Further upstream of the −10 region, separated by 19 bases, was a corresponding −35 region (TTGAAA) that conserves the most important part of the −35 consensus sequence (TTGNNN). Thus, the spiroplasmal RNA polymerase seems to conserve the DNA consensus sequences of the *E. coli* RNA polymerase. Very similar promoter structures were also reported for a tRNA gene cluster from *M. capricolum* (292), but direct evidence for this region functioning as a promoter in mycoplasmas is still missing. Rogers et al. (245) could not find sequences 3′ flanking to each of the seven tRNA genes in the spiroplasmal chromosome showing a dyad symmetry to fold into stem and loop structures for termination signal sequences. This led them to conclude that the tRNA gene cluster of *Spiroplasma* sp. strain BC-3 is transcribed as a single unit of at least seven tRNA genes and that the terminator region is located outside of the segment analyzed. More recently Yamao et al. (292), upon sequencing a *M. capricolum* DNA segment carrying a pair of tRNA genes, reported the presence of a probable termination signal after the genes. This signal consisted of a dyad symmetrical structure and a stretch of thymidine residues 24 bp downstream from the coding sequence for the second gene. Hence, the presumed mycoplasmal promoters and terminators resemble the classical procaryotic ones, a finding in line with their apparent recognition by the RNA polymerase of *E. coli* (see next section).

Unfortunately, although the complete sequences of the 16S rRNA genes of *M. capricolum* and of the related *Mycoplasma* sp. strain PG50 are now available (41, 78, 125) the segments sequenced did not contain enough of the 5′ flanking region to include the promoter(s). Analysis of this region is of particular interest because it may play an important role in stringent control and other control mechanisms of mycoplasmal rRNA gene expression (88).

Stringent control of stable RNA (rRNA and tRNA) synthesis is one of the best-known control mechanisms of gene expression. Wild-type *E. coli* deprived of a required amino acid shows a stringent response involving various alterations in the normal physiology of the cell, including the arrest of net synthesis of stable RNA, lipids, and peptidoglycan. The stringent response is found in cells of the $RelA^+$ (stringent) but not the $RelA^-$ (relaxed) phenotype. The intracellular concentration of guanosine 5′-diphosphate-3′-diphosphate (ppGpp) and guanosine 5′-triphosphate-3′-diphosphate (pppGpp) increases dramatically in amino acid-deprived $RelA^+$ cells due to the enzymatic activity of the *relA* gene product, which also stimulates ppGpp synthesis in vitro. The correlation between stringent response and increase in the intracellular concentration of ppGpp has been interpreted as evidence that this nucleotide has a causal role in stringency (30). *M. capricolum*, being one of the least-exacting mycoplasmas, was selected by Glaser et al. (88) for studies on stringent control mechanisms in mollicutes because this mycoplasma can be grown in a partially defined medium. Stable RNA synthesis in *M. capricolum* was found to be markedly affected by the omission of the amino acid supplement from the medium. The major conclusion drawn from these experiments was that synthesis of stable RNA in *M. capricolum* is subject to a stringent control mechanism resembling that described in other procaryotes (88). However, in contrast to the findings with *E. coli*, the major nucleotide accumulated in the mycoplasma was pppGpp rather than ppGpp. Detailed components involved in stringent control as well as the mechanisms and factors controlling the synthesis and accumulation of ppGpp and pppGpp are still unclear and under dispute. The fact that mollicutes contain only one, or at most two, sets of rRNA genes would seem to facilitate studies on stringent control, studies which in *E. coli* are complicated by the presence of seven sets of rRNA genes (see Ribosomal Ribonucleic Acid Genes section).

Expression of Cloned Mycoplasma Genes

The remarkable power of recombinant DNA techniques may be effectively used in the characterization of the small genome of mollicutes. The use of cosmid or Charon phages as vectors would, theoretically, enable the construction of a complete genomic library of a 700- to 800-kbp genome in about 200 clones, each containing 20-kbp inserts. Taylor et al. (273) used the λ Charon 4A phage as a vector for large segments of *M. hyorhinis* DNA produced by incomplete *Eco*RI digestion. Six recombinant phages, each containing distinct, nonoverlapping mycoplasmal genome segments of 10.3 to 16.0 kbp, cumulatively representing over 10% of the entire *M. hyorhinis* genome, were characterized (289). Thus, a substantial portion of the mycoplasma genome could be isolated in relatively few clones containing conveniently sized DNA sequences. The clones may serve as a starting point for a detailed analysis of pertinent genes within a segment and for the eventual delineation of the physical arrangements of the segments on the chromosome. The fact that the λ Charon 4A phage carries strong promoter regions flanking the recombinant sequences increases the probability of expression of mycoplasmal gene products in *E. coli* (273), although, as will be mentioned below, it appears that mycoplasmal promoters may also be active in the *E. coli* host.

Genomic libraries of mycoplasmal DNA were also prepared in the pBR family of *E. coli* plasmids. Thus, pBR325 was successfully used to clone rRNA genes of *M. capricolum* (7) and genes for protein antigens of a related mycoplasma, *Mycoplasma* sp. strain PG50 (8, 41). A variety of other vectors, which facilitate the detection of specific genes or control regions, have also been employed (cf. reference 116).

The cloned mycoplasmal genes were effectively expressed in *E. coli*. Thus, immunological screening of recombinant libraries by monoclonal antibodies or by polyclonal antisera, employing immunoblotting or immunoprecipitation techniques, could identify a variety of mycoplasma protein antigens produced in the *E. coli* host (41, 183, 273, 289). The use of *E. coli* minicells or maxicells as hosts for recombinant phages or plasmids facilitates the identification of specific mycoplasmal antigens (8, 41, 130, 289). Spiralin, a major protein antigen of *S. citri*, was produced in *E. coli* by transfection of the bacterium with pBR328 containing a 6.5-kb insert of *S. citri* DNA (183). However, the main form of spiralin produced in *E. coli* had a somewhat higher molecular weight (30.5 kDa) than the spiralin produced in *S. citri* (28 kDa). This led Mouches et al. (183) to propose that due to inefficient posttranslational processing, spiralin is expressed in *E. coli* as a preprotein containing a signal peptide. Moreover, the prespiralin produced in *E. coli* accumulated mainly in the cytoplasm, whereas in *S. citri* the processed spiralin is located in the cell membrane, probably spanning it. The cytoplasmic location of the prespiralin in *E. coli* facilitated the isolation of the protein by immunoaffinity chromatography on Sepharose beads coated with antispiralin antibodies (183). Klinkert et al. (136), on the other hand, failed to obtain autonomous expression in *E. coli* of *M. hyopneumoniae* genes from large DNA fragments (5 to 10 kb) cloned either in pBR322 or in a phage λ vector. They prepared a genomic library of the mycoplasma in the plasmid pEx29, which expresses foreign DNA sequences fused to a gene that is highly expressed in *E. coli*, as it makes use of the PL promoter of phage λ to provide efficient transcription of foreign DNA inserts. In this way, Klinkert et al. (136) succeeded in cloning short random fragments of *M. hyopneumoniae* DNA coding for one or more epitopes of surface antigens of this mycoplasma.

Can the *E. coli* transcription machinery recognize mycoplasmal promoters? The answer appears to be positive. Thus, reversed orientation in the vector of the spiroplasmal DNA insert with the spiralin gene did not affect its expression in *E. coli*. Furthermore, use of a pBR328 plasmid with a deleted tetracycline-resistance (Tc^r) promoter did not affect expression of the spiralin gene either (183). Wise (289) also mentions that insertion into the vector of *M. hyorhinis* DNA segments in opposite orientation resulted in the synthesis of similar proteins in *E. coli*. At least six genes for *M. capricolum* ribosomal proteins were shown by Kawauchi et al. (130) to be expressed in *E. coli* from their own promoter(s). Thus, it appears that *E. coli* RNA polymerases can recognize the transcription signals of mycoplasmal genes. A similar conclusion appears to hold also for promoters in DNA of the *A. laidlawii* MVL2 virus cloned into an *E. coli* plasmid (116).

Production of vaccines made of selected immunogens rather than of whole organisms has been one of the major trends in vaccine development (14). Identification of *M. pneumoniae* membrane proteins responsible for adherence of this respiratory pathogen to tracheal epithelium encourages the development of a specific vaccine made of these proteins (222). However, the low yields of *M. pneumoniae* and the complex and expensive media needed for its cultivation prohibit the use of the mycoplasmas themselves as a source for these proteins. Clearly, the ability to clone the specific mycoplasma genes and obtain their expression in *E. coli* is expected to open the way for large-scale production of mycoplasmal antigens for research and vaccine development (136).

RIBOSOMES

Ribosomal Proteins

Mycoplasmal ribosomes resemble typical eubacterial ribosomes in having a sedimentation coefficient of about 70S, three rRNA species (5S, 16S, and 23S), and about 50 protein species. Recent 2D-PAGE analysis by Kawauchi et al. (129) revealed 30 protein spots in the 50S subunits and 21 protein spots in the 30S subunits of *M. capricolum* ribosomes, corresponding to the number of proteins in eubacterial ribosomes. Moreover, *M. capricolum* ribosomal proteins resembled those of eubacteria in size and electrophoretic mobility, the resemblance being most pronounced with ribosomal protein profiles of gram-positive bacilli (129).

An interesting, although somewhat expected, conclusion is that the genes for ribosomal proteins are well conserved in the mycoplasma genome despite its smaller genetic capacity. Thus, a significant portion of the *M. capricolum* genome is encoding ribosome synthesis (about 55 genes of an estimated total of 400 to 600 genes). A recombinant plasmid carrying a cluster of genes for at least eight *M. capricolum* ribosomal proteins was recently constructed by Kawauchi et al. (130). The protein genes, clustered apparently as an operon, were expressed in *E. coli* from their own promoter. A 1.3-kbp segment of the recombinant plasmid DNA carrying the genes for ribosomal proteins S8 and L6 and a part of L18 was sequenced (190). The A+T content of the *M. capricolum* genes was 71 mol%, much higher than that of the corresponding *E. coli* genes (49 mol%). Comparison of codon usage between the two organisms revealed that *M. capricolum* preferentially uses A- and U-rich codons (U, uracil). The biased choice of the A- and U-rich codons has also been observed by Muto et al. (190) in the codon replacements for conservative amino acid substitutions between *M. capricolum* and *E. coli*. As will be discussed in the section on rRNA genes, the preference for A and T was also noticed for the spacers between the rRNA genes. Thus, it appears that the constraint for the preferential use of A and T in the A+T-rich *M. capricolum* (76 mol%) has operated at the DNA level as a selection force upon the codon choice as well as on the construction of other parts of the genome.

Ribosomal Ribonucleic Acids

The structure of the smallest rRNA species, the 5S rRNA, has become a subject of extensive research since Hori and Osawa (118) showed that these molecules can serve as phylogenetic markers. Nucleotide sequence analysis of these relatively small molecules can be accomplished fairly easily. According to length, the eubacterial 5S rRNAs are classified into two major types: one, with 120 nucleotides, characterizes gram-negative bacteria, and the other, 116 to 117 nucleotides long, characterizes the gram-positive bacilli and clostridia (118). The eucaryotic 5S rRNA is 120 nucleotides long but differs from procaryotic 5S rRNA types in having a well-conserved loop at positions 83 to 94 and in lacking the hairpin structure. Archaebacterial 5S rRNAs

show some peculiar properties. Thus, the *T. acidophilum* 5S rRNA, being 122 nucleotides long, possesses a mixture of the characteristic features of both eubacterial and eucaryotic 5S rRNAs (160), while the 5S rRNA homolog of *Halococcus morrhuae* is 231 nucleotides long, possessing a 108-nucleotide segment in its interior not homologous to any region of a normal-size 5S rRNA (161).

Based on data for 13 different mollicutes, the 5S rRNAs of these organisms appear to be definitely shorter than any 5S rRNA species recorded so far. The length of the mollicutes 5S rRNA ranges from 113 nucleotides in *Anaeroplasma* species down to 104 nucleotides in *Ureaplasma urealyticum* (119, 244, 284). The shorter lengths are due to small deletions in the regions of the 5S rRNAs comprising helix E-E′ and in the single-stranded loop formed by helix E, indicating that the 5S rRNA molecule can tolerate considerable perturbation in this region and still function (196, 244). The 5S rRNA of *M. capricolum* also has the lowest G+C content (42 mol%) among the 5S rRNAs yet sequenced, in accord with the extremely low G+C content of the genome of this mycoplasma (119). In addition, modified nucleotides were not present in mycoplasmal 5S rRNAs (119, 284). As will be discussed in the Phylogeny section, the primary structures of the mycoplasmal 5S rRNAs are more related to those of gram-positive bacteria than to those of gram-negative bacteria. Within the *Mollicutes*, the 5S rRNAs of related species were very similar. Thus, the nucleotide sequence of the 5S rRNA of *M. mycoides* subsp. *capri* (284) showed only three differences from the sequence of *M. capricolum* 5S rRNA (119).

Our rather limited knowledge of the structure of mycoplasmal 16S rRNAs comes mostly from oligonucleotide catalogs (290, 291) and from sequencing of several cloned genes of this rRNA species, as will be discussed in the section on rRNA genes. As to mycoplasmal 23S rRNAs, information is even more scarce. In size, the mycoplasmal 23S rRNAs resemble those of eubacteria (234), but as expected, differences in nucleotide sequences must exist. Thus, a segment of the 23S rRNA gene of *M. hyorhinis* failed to hybridize with the corresponding segment of the 23S rRNA gene of *E. coli* at 65°C (90). Physical maps of rRNA operons of various mollicutes also show differences in restriction sites in the region of the 23S rRNA gene (Nur et al., submitted for publication).

Synthesis and Methylation of Ribosomal Ribonucleic Acids

As was mentioned above (Gene Expression section), rRNA synthesis in *M. capricolum*, and possibly in other mycoplasmas, is under stringent control. Since mycoplasmas very frequently contaminate cell cultures used in studies on mitochondrial rRNA, a comparison has been made by Harley and Rees (113) of rRNA synthesis in mitochondria and mycoplasmas. Mycoplasmal and mitochondrial rRNA species appear to share several properties, such as low G+C content, low order of base methylation, and inhibition of their synthesis by low concentrations of actinomycin D. However, mycoplasmal rRNA synthesis is resistant to low concentrations of ethidium bromide, sufficient for specific inhibition of mitochondrial rRNA synthesis. Furthermore, individual mycoplasmal rRNA species are quite distinct from the mitochondrial rRNA species (113). In fact, recent hybridization data with cloned rRNA genes of mollicutes indicated little, if any, nucleotide sequence homology with the corresponding mitochondrial rRNA genes (90, 228). This stands in contrast to the recent finding of high homology of rRNA genes of mollicutes and plant chloroplasts (I. Nur and S. Razin, unpublished data).

The low order of methylation of mycoplasmal rRNAs has also been noticed by Hsuchen and Dubin (122). The methylation patterns of *M. capricolum* and *A. laidlawii* 16S rRNA were typically procaryotic, retaining the methylated residues previously shown to be highly conserved among eubacterial 16S rRNAs. The mycoplasmal 23S rRNA methylation patterns were, on the other hand, rather unusual. *M. capricolum* 23S rRNA contained only four methylated residues in stoichiometric amounts, all of which were ribose methylated. *A. laidlawii* 23S rRNA contained the same methylated ribose residues, plus approximately six 5-methyluridine residues (122).

Ribosomal Ribonucleic Acid Genes

The best-characterized mycoplasmal genes are those encoding for rRNAs. There are several reasons for focusing the attention on these genes. (i) rRNA genes and their products are highly conserved and thus serve as effective phylogenetic markers (see Phylogeny section). (ii) It is easy to select and clone these genes. (iii) Cloned rRNA genes can serve as probes for detection and identification of mollicutes in clinical material (see Diagnostic Probes section). (iv) The finding that many mycoplasmas have only one set of rRNA genes may facilitate studies on control mechanisms of rRNA synthesis (see Gene Expression section).

The first mycoplasmal rRNA genes to be studied were those of *M. capricolum* (7, 255). The choice fell on this mycoplasma because the early work of Ryan and Morowitz (250) suggested that the *M. capricolum* genome carries only one set of rRNA genes. This assumption proved to be wrong, since the more recent studies of Sawada et al. (255), using Southern hybridization of restriction enzyme digests of *M. capricolum* DNA with [^{32}P]-labeled 16S, 23S, and 5S rRNAs, have indicated the presence of two sets of genes for the three rRNA species. At about the same time Amikam et al. (7) succeeded in cloning in pBR325, a 4.8-kbp segment of the *M. capricolum* genome carrying the entire genes for the 23S and 5S rRNAs and part of the 16S rRNA gene. The recombinant plasmid, named pMC5, yielded seven hybridization bands with *E. coli* DNA digested by *Bam*HI, an enzyme known to cleave only in the spaces between the seven rRNA operons of this bacterium. This result indicated a high degree of sequence homology between the rRNA genes of *M. capricolum* and the corresponding genes of *E. coli* and opened the way for using defined segments of the rRNA operons of *E. coli* as probes to study the mode of arrangement of the rRNA genes in mollicutes. The plasmid pKK3535, containing the entire *rrnB* operon of *E. coli* (28), was used for this purpose. Since the entire nucleotide sequence of this operon has been established (28), it was possible to prepare defined segments of it to serve as differential probes (6, 87, 90, 227).

Hybridization data and the resulting physical maps obtained with the various probes (Fig. 2) indicated that the rRNA genes in all the mollicutes tested by us (6, 7, 87, 225, 227, 228) and by others (78, 89, 125, 194, 254, 255) are linked in the classical order found in procaryotes, that is, 5′-16S-23S-5S-3′. Moreover, the three genes are close to each other, occupying a chromosomal segment of about 5 kbp, suggesting that the three genes function as a single transcriptional unit, that is, an operon. Interestingly, this does not appear to apply to *T. acidophilum*, the wall-less archaebacterium, once included in *Mollicutes*. In this bacterium,

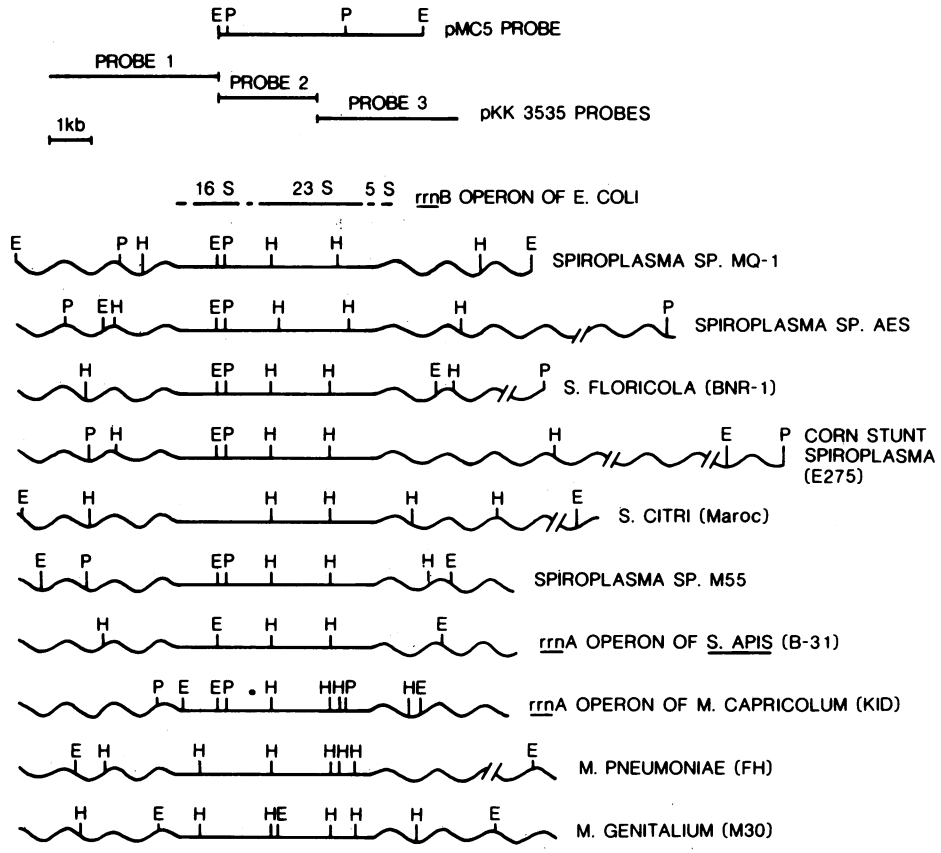

FIG. 2. Physical maps of rRNA operons of various mollicutes. Restriction endonucleases: E, *Eco*RI; P, *Pst*I; H, *Hin*dIII (I. Nur, I. Ehrlich, D. Yogev, and S. Razin, unpublished data).

large spacer regions of at least 6 and 7.5 kbp were found to separate the three rRNA genes (279).

Hybridization performed with pMC5 and the pKK3535 rRNA probes enabled an estimation of the number of rRNA genes in the mycoplasma genome. Recent surveys (6, 7, 89, 194, 255) indicate that the genome of mollicutes carries only one or two copies of rRNA genes. In the several eubacteria examined so far, a multiplicity of rRNA genes was found. Thus, the *E. coli* genome carries seven copies of the genes for the 16S and 23S rRNAs and eight copies of the 5S rRNA gene (134) while *B. subtilis* carries at least 10 copies of each of the three genes (159) and *Streptococcus cremoris* and *Streptococcus lactis* carry at least five or six sets of rRNA genes (194). The low number of RNA genes in mollicutes is in line with the concept of economy in genetic information in microorganisms with a small genome (176, 177). However, there seems to be no strict correlation between genome size and number of rRNA genes. Thus, the *M. arginini* genome contains two copies of the rRNA genes, while *S. citri*, with a genome of twice the size, contains only one copy of the genes (6). Furthermore, the archaebacterium *Halobacterium halobium* contains only one set of rRNA genes, despite its genome size being close to that of *E. coli* (114). The fact that the free-living archaebacteria *Halobacterium halobium* and *T. acidophilum* possess only one set of rRNA genes (114, 279) does not support the idea that the low number of rRNA genes in mollicutes reflects their parasitic mode of life. Nevertheless, one cannot discount the possibility that the low number of rRNA genes in mollicutes is a result of extensive genetic deletions during their presumed evolution from eubacterial ancestors (see Phylogeny section). Clearly, the large number of rRNA operons in the eubacteria *E. coli* and *B. subtilis* may serve to amplify rRNA synthesis and facilitate extremely rapid cell growth and reproduction, a feature not shared by mollicutes.

Since the number of rRNA operons in mollicutes is only one or two and there is either one or no *Eco*RI site in the entire operon (6, 89), the Southern hybridization patterns obtained with pMC5 or the pKK3535-derived probes are very simple and consist of one to four bands (Fig. 3) depending on the number of operons (one or two), the presence or absence of an *Eco*RI site in the operon, and the type of probe used (covering the proximal, central, or distal parts of the operon). Thus, in *M. pneumoniae* or *S. citri* there are no *Eco*RI sites along the entire rRNA operon resulting in a single hybridization band. Yet the size of the segment hybridized differs in *M. pneumoniae* and *S. citri* because the flanking sequences on both sides of the operon are different in the two organisms. The characteristic number and size of the hybridization bands obtained with each of the mollicutes enable the identification of mycoplasmal DNA with the specific rRNA probes (see section on Diagnostic Probes).

The physical maps of the rRNA operons of different mollicutes (87, 89, 189) (Fig. 2) indicate some differences in nucleotide sequences in homologous rRNA genes, although some restriction sites such as the *Eco*RI site at the proximal end of the 16S rRNA gene and the *Hin*dIII sites in the 23S rRNA gene appear to be rather common, while other restriction sites are more specific.

Elucidation of the mode of organization of mycoplasmal rRNA genes has been recently followed by complete se-

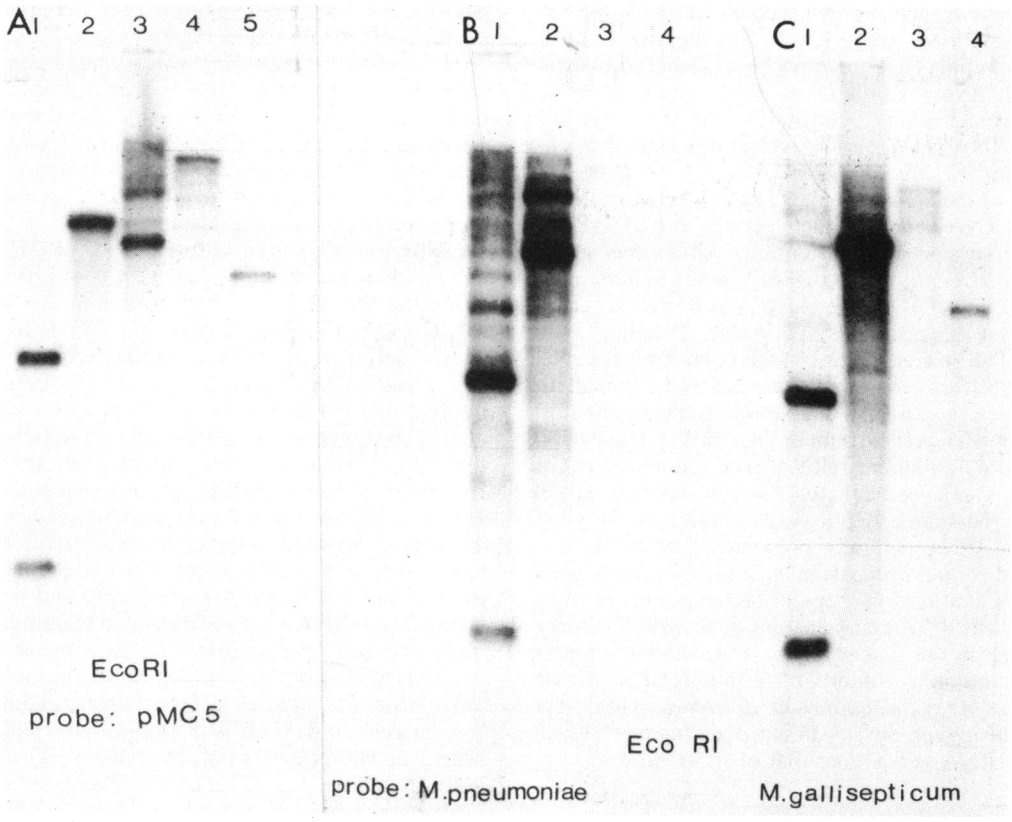

FIG. 3. Hybridization patterns of *Eco*RI-digested DNAs of *M. genitalium* (lanes 1), *M. pneumoniae* (lanes 2A, 2C), *M. gallisepticum* (lanes 3A, 2B), *S. citri* (lanes 4A, 3B, 3C), and *M. pulmonis* (lanes 5A, 4B, 4C) with the ribosomal DNA probe pMC5 (containing the rRNA genes of *M. capricolum*) and with nick-translated ^{32}P-labeled total DNAs of *M. pneumoniae* and *M. gallisepticum* as probes. The bands of rRNA genes revealed by pMC5 can also be seen with the total DNA probes. However, the *M. pneumoniae* total-DNA probe reveals additional bands upon interaction with digested DNAs of *M. genitalium* and *M. gallisepticum*, indicating the presence of common genes. The *M. gallisepticum* total-DNA probe reveals common genes with *M. pneumoniae* but very few bands additional to the rRNA genes upon interaction with *M. genitalium*. Both total-DNA probes reveal only the rRNA genes in *S. citri* and *M. pulmonis* (D. Yogev, I. Nur, and S. Razin, unpublished data).

quencing of the 16S rRNA gene of *M. capricolum* (125) and the related *Mycoplasma* sp. strain PG50 (41, 78). The 16S rRNA gene of *M. capricolum* is 1,521 bp long, that is, 21 bp shorter than that of *E. coli* and 34 bp longer than that of *Anacystis nidulans* (125). The mycoplasmal gene shows a remarkable sequence homology to that of *E. coli* (74% identity) and to *A. nidulans* (76% identity). These homology values are considerably higher than those obtained on comparing the 5S rRNA of *M. capricolum* and *E. coli* (56% identity) or *A. nidulans* (53% identity). Yet, the G+C content of the *M. capricolum* 16S rRNA gene is 48 mol%, a value lower than that of the corresponding gene of *E. coli* (55 mol%) and of *A. nidulans* (56 mol%). One of the two 16S rRNA genes of *Mycoplasma* sp. strain PG50 has been sequenced by Frydenberg and Christiansen (78). The data resemble very much those obtained with *M. capricolum*. The PG50 16S rRNA gene is 1,523 bp long, has a G+C content of 49 mol%, and shows about 70% sequence homology to the *E. coli* 16S rRNA gene. It should be stressed that the two 16S rRNA genes of *Mycoplasma* sp. strain PG50 are not identical, as one of them has a *Hin*dIII site not found in the other (41). Hybridization data obtained with *M. gallisepticum* A5969 were likewise taken to suggest nucleotide sequence differences between the two rRNA operons of this strain (6). Minor differences among homologous genes carried on the same genome are apparently not rare; thus 5 of the 10 rRNA gene clusters in *B. subtilis* lacked a *Hin*dIII site at their 3′ ends (159).

The secondary structure of the *M. capricolum* 16S rRNA gene constructed according to the models for the *E. coli* 16S rRNA gene revealed a striking similarity. Almost all the stem and loop structures in the *E. coli* gene could be observed in the *M. capricolum* gene, indicating a strong conservation of the secondary structures of these two bacterial 16S rRNAs (125). Nevertheless, detailed comparative analysis of the secondary structure of the 16S rRNA genes of *Mycoplasma* sp. strain PG50, *E. coli*, and *Bacillus brevis* (78) revealed some differences in the stems while the loops remain conserved. The PG50 gene sequence resembled more the *B. brevis* than the *E. coli* gene sequence in many of the variable regions, an observation supporting the closer relatedness of mycoplasmas to gram-positive bacteria (see Phylogeny section).

The 3′-terminal region of the *M. capricolum* 16S rRNA gene contained the sequence 5′-ACCTCC-3′ (125), a proposed procaryotic mRNA-binding sequence (257). A similar Shine and Dalgarno sequence could also be observed at the 3′ terminus of the PG50 gene, but the extreme 3′ terminus of the mycoplasmal gene resembled that of the *B. brevis* gene and differed from that of *E. coli* (78). Sequence differences at the extreme 3′ terminus of 16S rRNA genes have been invoked as a cause for the failure of *B. subtilis* ribosomes to

translate effectively from *E. coli* messenger RNAs (175). Yet the *E. coli* translation system can recognize the start of translation of mycoplasmal mRNA (see Gene Expression section). Nonetheless, it would be of interest, in light of the greater similarity of the 3′ terminus, to test whether mycoplasmal mRNA is more effectively translated in *Bacillus* species than in *E. coli* (78).

The structure of the spacer regions located between the 3′ end of the 16S rRNA gene and the 5′ end of the 23S rRNA gene has attracted particular attention. All of the seven rRNA operons of *E. coli*, the two rRNA gene clusters in *A. nidulans*, and 2 of 10 rRNA gene clusters in *B. subtilis* carry one or two tRNA genes in this space (for references, see reference 125). The spacer regions in the two rRNA operons of *M. capricolum* were estimated to be 228 and 226 nucleotides long, respectively, and revealed 97% sequence homology. These regions were extremely rich in A+T (over 80 mol%) and did not contain any tRNA gene sequences (125). Similar findings were reported for *Mycoplasma* sp. strain PG50, although the spacer region was estimated to be only 209 bp long (41). It is generally presumed that the spacer sequence includes the processing signals of rRNA gene transcripts in the maturation steps of rRNA synthesis. The finding that 8 of 10 rRNA gene clusters of *B. subtilis* do not carry tRNA genes in the spacer region (159) and the remarkable sequence homology found in certain regions of the spacers between *M. capricolum* and *B. subtilis* (125) suggests that *M. capricolum* possesses a processing mechanism independent of tRNA genes, like that of *B. subtilis*.

Transfer Ribonucleic Acid

tRNAs, like rRNAs, are highly conserved molecules with respect to size, composition, and function. Being relatively small, tRNA molecules were among the first mycoplasmal macromolecules to be sequenced. Much of the recent interest in mycoplasmal tRNA stems from the phylogenetic information that can be derived from their structural studies (283). The rather extensive studies on mycoplasmal tRNAs carried out in the late 1960s and early 1970s summarized in previous reviews (218, 261) have revealed several interesting properties. (i) The low G+C content of the mycoplasma genome is not reflected in the base composition of the mycoplasmal tRNAs. Thus, the G+C ratios of mycoplasmal tRNAs were found to be in excess of 50 mol%, resembling the values for eubacterial tRNAs. Furthermore, the secondary structure of mycoplasmal tRNA fits the familiar cloverleaf model, in line with the highly conserved nature of these molecules. (ii) Mycoplasmas appear to lack many isoaccepting tRNA species. For example, *M. mycoides* subsp. *capri* contains only one tRNA species for each of the amino acids glycine, lysine, valine, and methionine (132, 285), while *M. capricolum* contains only one tRNA species for phenylalanine. It is tempting to speculate that the small size of the genome and its extremely low G+C content were evolutionary factors pressuring to decrease the number of nonessential gene products, such as isoaccepting tRNA species. The finding that in mitochondria, where genome size is extremely small, all codons are read by 24 tRNA species only, compared with around 50 or more tRNA species found normally in cells, supports the above notion (132). (iii) Mycoplasmal tRNAs are poor in modified nucleosides (218, 261). The glycine tRNA ($tRNA^{Gly}$) of *M. mycoides* subsp. *capri* contains three modified nucleosides only (132). The lack of certain modified nucleosides from mycoplasmal tRNAs argues against their being essential for the proper functioning of tRNAs (for references see reference 218).

M. mycoides subsp. *capri* was found to contain the typical eubacterial initiator tRNA, formylmethionine tRNA ($tRNA^{fMet}$), although with fewer modified nucleosides (285). However, the closely related *M. mycoides* subsp. *mycoides*, incapable of folate synthesis, is still able to grow in the absence of folate derivatives. It seems, therefore, that under these conditions the organisms must initiate protein synthesis with nonformylated Met-$tRNA^{fMet}$ (192). In *Streptococcus faecalis*, for which a similar situation has been reported (252), the Met-$tRNA^{fMet}$ had uridine in the sequence -G-U-ψ-C-G- in loop IV. When folate is available the uridine at this site is methylated to give the usual sequence -G-T-ψ-C-G-, and formylation must occur to allow initiation. It is of interest that the $tRNA^{fMet}$ of *M. mycoides* subsp. *capri* has also been shown to have uridine rather than ribothymidine at this site (285). If a similar situation occurs in the closely related *M. mycoides* subsp. *mycoides* it could account for its ability to initiate protein synthesis without an fMet tRNA.

Despite the small number of nucleotides in tRNAs their sequencing is not easy, since RNA sequencing is generally more difficult than DNA sequencing, and the tight tertiary structure of tRNAs and the presence of many modified bases make the task even harder. Hence, the recent cloning of mycoplasmal tRNA genes is expected to facilitate considerably structural studies of the corresponding tRNAs. Although genetic data are still too scarce to allow generalizations, the tRNA genes that have been identified thus far in both *M. capricolum* (292) and *Spiroplasma* sp. strain BC-3 (245) are organized in clusters, transcribed apparently as single units. The intergenic regions are very rich in A+T (over 80 mol%), while the genes themselves have a G+C content of about 55 mol%, normal for tRNA genes and considerably higher than the G+C content of the genome. This finding, shown also to be true for intergenic regions in mycoplasmal rRNA operons (see section on rRNA Genes) must reflect the necessity of retaining essential G+C base pairs for the tRNA and rRNA transcripts to conserve functionality (245). It should be recalled here that no tRNA genes could be found in the spacer regions between the 16S and 23S rRNA genes of *M. capricolum* (189, 254) in contrast to the findings for rRNA operons of *E. coli* and several other eubacteria (see section on rRNA Genes).

The tRNA gene cluster of *Spiroplasma* sp. strain BC-3 cloned and characterized by Rogers et al. (245) consisted of $tRNA^{Cys}$-$tRNA^{Arg}$-$tRNA^{Pro}$-$tRNA^{Ala}$-$tRNA^{Met}$-$tRNA^{Ile}$ and a portion of $tRNA^{Ser}$ (Cys, cysteine; Arg, arginine; Pro, proline; Ala, alanine; Ile, isoleucine; Ser, serine). All the CCA-3′- terminal sequences were encoded in the genes. An RNA polymerase promoter site was found upstream of the $tRNA^{Cys}$ gene, but no obvious sequences 3′ flanking to the tRNA genes showing dyad symmetry to fold into stem and loop structures for termination signals could be found. Thus, it appears that the tRNA gene cluster is transcribed as a single unit of at least seven tRNA genes. The spiroplasmal tRNA gene cluster had no neighboring 5S rRNA gene close to its 5′ end, suggesting that this gene cluster is not immediately associated with an rRNA gene set as is the normal pattern in the *B. subtilis* genome (245). A very interesting finding has recently been reported by Yamao et al. (292). A 600-bp subfragment of a 2.0-kb segment of *M. capricolum* DNA cloned in pBR322 was found to carry a pair of tRNA genes, with a 40-bp spacer between them. The tRNA encoded by the first gene has an anticodon sequence 5′-U-C-A-3′ that can decode both opal codon UGA and universal

tryptophan codon UGG, whereas the second one has an anticodon sequence 5'-C-C-A-3' for the universal UGG codon for tryptophan. It should be recalled that UGA is considered a nonsense or termination (opal) codon throughout procaryotes and eucaryotes. The finding that UGA is used by *M. capricolum* as a tryptophan codon may therefore be of great phylogenetic interest, particularly as this property is shared by mitochondria (see Phylogeny section). Evidence for the use of UGA as a tryptophan codon in *M. capricolum* was obtained by sequencing *M. capricolum* genes coding for the ribosomal proteins S3 and S16, which are highly homologous to S3 and S16 of *E. coli*. The mycoplasmal genes contained four UGA codons in the reading frames. Three of these occurred at positions corresponding to tryptophan in the homologous *E. coli* proteins (292).

The construction of the spiroplasmal tRNA genes in the cloverleaf form enabled their comparison to homologous tRNA genes of eubacteria. The conclusion was that the spiroplasmal genes show higher sequence homology to the corresponding genes of *B. subtilis* than to those of *E. coli*, providing further evidence for evolution of mycoplasmas from gram-positive eubacteria (245).

CLONED GENES AS DIAGNOSTIC PROBES

The frequent difficulty encountered in cultivating mycoplasmas hampers the laboratory diagnosis of mycoplasma infections. In extreme cases, such as plant and insect infections by the uncultivable mycoplasma-like organisms (MLO), diagnosis by conventional techniques is not possible. The extremely slow growth from clinical specimens of *M. pneumoniae* and more so of the newly discovered *M. genitalium* (281) puts the weight on serological techniques that suffer from being relatively nonspecific (18, 123, 131, 148, 276). Detection and identification of mycoplasmas infecting cell cultures constitutes another difficult problem. *M. hyorhinis*, a common cell culture contaminant, very frequently fails to grow in conventional culture media (117) so that diagnosis must rely on indirect methods, such as DNA staining, enzymatic reactions, specific antibodies, etc. (280). All of these indirect means suffer from a variable degree of nonspecificity and low sensitivity.

A new approach to laboratory diagnosis of infections is based on gene probes. Genes specific for a particular group or a single species of infectious agents are selected, cloned, and used as probes in hybridization tests with DNA of the infected tissue or clinical specimen. Although studies in this area are still in their infancy, two classes of probes can already be distinguished. One consists of rRNA genes and the other of genes coding for mycoplasma proteins.

Ribosomal Ribonucleic Acid Gene Probes

As was discussed in detail in the section on rRNA genes, these highly conserved genes show significant sequence homology among the *Mollicutes*. Moreover, the cloned rRNA genes of *M. capricolum* react positively in hybridization tests with the corresponding genes of *E. coli* and vice versa (6, 7, 89, 90), suggesting that these may serve as general probes for procaryotes. However, the finding that rRNA operons in mollicutes frequently differ in restriction sites within the operon and have different flanking sequences enables the use of these genes as probes for identifying specific mycoplasmas. Thus, Southern blot hybridization of cloned *M. capricolum* rRNA genes with DNAs of various mycoplasmas digested by restriction endonucleases results in hybridization patterns specific for each of the tested mycoplasma strains (6, 7, 225, 227). This principle has been successfully applied to the detection and identification of mycoplasmas in contaminated cell cultures (227, 228), using as a probe the recombinant plasmid pMC5 containing the entire 23S and 5S and most of the 16S rRNA genes of *M. capricolum* (7, 87). DNA of mycoplasma-free cell cultures did not hybridize with the probe, as the eucaryotic rRNA genes show very little, if any, homology with their procaryotic counterparts (228). The method employed by Razin et al. (228) was capable of detecting the presence of less than 10^5 mycoplasmas per ml of culture, a level lower than that found in most contaminated cultures. Furthermore, this procedure enabled the identification of the contaminating mycoplasma. The efficacy of pMC5 as a probe for detecting mycoplasmas has been confirmed by Taylor et al. (274, 275) using the rapid dot hybridization technique. However, the mycoplasma species cannot be identified by this technique.

More recently, Göbel and Stanbridge (90) succeeded in developing a mycoplasma rRNA gene probe which would not interact with *E. coli* DNA under certain hybridization conditions and will thus probably be specific for mycoplasmas. The probe consists of a 0.9-kbp segment obtained from the 5'-terminal region of the *M. hyorhinis* 23S rRNA gene by *Hin*dIII digestion. This segment hybridizes with the *E. coli rrnB* operon at 55°C but not at 65°C. Cloning of this segment into the bacteriophage M13 provided a probe that hybridized well at 65°C with mycoplasmal DNA but showed negligible cross-hybridization to *E. coli* DNA at this temperature and no cross-hybridization at all to chromosomal or mitochondrial DNA of HeLa cells. A dot-blot hybridization procedure with this probe could detect fewer than 10^5 mycoplasmas, ranking with the most sensitive indirect methods for mycoplasma detection (90). Hence, this probe, depending on hybridization conditions, may be used to detect any procaryotic organism, or specifically mycoplasmas. Nevertheless, the rapid dot-blot test cannot identify the mycoplasma species.

Protein Gene Probes

Obviously, the chances of cloned protein genes serving as species-specific probes are much greater than those of the highly conserved rRNA genes. The recent work by Taylor et al. (274, 275) supports this assumption. Two cloned DNA fragments from a genomic library of *M. hyorhinis* demonstrated species-specific dot hybridization tests with DNAs from a panel of different mycoplasmas. These probes selectively recognized *M. hyorhinis* sequences in purified DNA, broth-grown organisms, and infected cell cultures. The dot-blot hybridization test employed was extremely sensitive, enabling the detection of 5×10^4 *M. hyorhinis* organisms from broth culture deposited directly on the nitrocellulose sheet. Similar direct blot analysis of washed murine lymphoblastoid cells infected with *M. hyorhinis* was capable of detecting cell-associated mycoplasmas even when less than 20% of the cell population was infected, with no background hybridization to the eucaryotic DNA (274, 275).

The success of Taylor et al. (274, 275) in developing a specific gene probe for *M. hyorhinis* would support the search for similar probes, each specific for a different species. This requires the construction of genomic libraries and the selection of clones which will react only with the desired species. Taylor et al. (274, 275) comment that total genomic

DNA hybridization tests with several mycoplasmas have demonstrated limited homology, even among serologically related species, facilitating the isolation of discrete segments of the mycoplasma genome capable of serving as species-specific probes. Our recent experience with total genomic mycoplasmal DNA as probes in Southern blot hybridizations, although generally confirming the above statement, calls for some caution, particularly with the pair *M. pneumoniae-M. genitalium*. Southern blot hybridization of digested DNA of one organism with nick-translated ^{32}P-labeled total DNA of the other revealed, in addition to the characteristic hybridization bands of the rRNA genes, other bands apparently of genes coding for common proteins (D. Yogev, I. Nur, G. Glaser, and S. Razin, submitted for publication; Fig. 3). This finding is in line with common antigenic determinants detected by Western immunoblots of cell proteins of these two mycoplasmas (18, 123, 131). Similar findings were recently reported for another pair of related mycoplasma species, *M. hyopneumoniae* and *M. flocculare* (32). Thus, the selection of species-specific probes from genomic libraries of mycoplasmas requires careful checking to prevent the picking up of DNA segments containing common genes. It should be mentioned in this context that the use of total genomic DNA as a probe can be employed for the identification of the mycoplasma species according to the characteristic hybridization patterns produced by the rRNA genes included in the total genomic probes, replacing in this way the need for specific rRNA probes.

Another trial to employ a protein gene as a diagnostic probe yielded peculiar and still unexplained results. Spiralin is a major protein for *S. citri*. Yet a cloned, 6.5-kbp *S. citri* DNA fragment containing the spiralin gene hybridized with one or several restriction fragments of the DNA of all other spiroplasma species tested by Mouches et al. (183) and even with DNA of *A. laidlawii* and *M. mycoides*. It thus appears that the cloned DNA fragment of *S. citri* contained in addition to the spiralin gene sequences common to other mollicutes. Whether these belong to the common rRNA genes is not clear. Nevertheless, this probe, when applied to DNA of periwinkle (*Vinca rosea*) plants infected with MLOs did not yield any hybridization bands, while it reacted positively in dot-blot tests with the DNA of the same plants infected with *S. citri* (183), a finding which does not support the identification of common sequences in the probe with rRNA genes.

Preliminary experiments in our laboratory (I. Nur, S. Razin, C. Saillard, and J. M. Bove, unpublished data) show that *Hin*dIII-digested DNA of *V. rosea* plants infected with different MLOs and *S. citri* react in Southern blot hybridization tests with the rRNA gene probe pMC5. The hybridization patterns were made up of two to three heavy bands and a few weaker bands. The heavy bands, identical in all tested samples, including those of control uninfected plants, were shown by us to represent chloroplast rRNA genes. Chloroplast rRNA genes exhibit high sequence homology with the corresponding rRNA genes of *E. coli* (144) and, as could be expected, with the rRNA genes of *M. capricolum*. The weaker bands appeared only in the infected-plant samples, and their pattern differed according to the infectious agent. The small number of bands may relate the MLOs to mollicutes. It is hoped that the patterns of hybridization bands will be useful in distinguishing the uncultivable MLOs.

The successful application of gene probes for the detection and identification of mycoplasmas in infected cell cultures attracts attention to the possibility of using similar probes in the detection and identification of mycoplasmas in clinical material from humans and animals. A pointer in this direction is provided by the recent study of Chan and Ross (32). A 9-kbp DNA segment of the swine respiratory pathogen *M. hyopneumoniae* cloned in pBR322 reacted positively in Southern blots with cleaved DNA prepared from lung washings of *M. hyopneumoniae*-infected pigs. Obviously, for testing material which may contain a variety of bacteria, specific probes are required, while rRNA gene probes may only be adequate for testing specimens in which only one type of organism is expected, such as in cerebrospinal fluid or blood.

In summary, probes made up of conserved genes such as the rRNA genes do offer the advantage of identifying and distinguishing multiple species with a single labeled reagent. For identifying species by these probes a complex hybridization procedure, involving DNA purification, digestion, electrophoresis, and Southern blot hybridization, is required. Specific gene probes, on the other hand, can identify specific mollicutes by the much simpler dot hybridization techniques. However, for this purpose a battery of reagents, each specific for a different species, is needed. Consequently, the two classes of probes may be considered as complementing each other. The way now appears open for the development of diagnostic kits made of cloned mycoplasma genes. An essential step toward this goal is the possible replacement of the ^{32}P-labeled probes with ^{3}H-labeled probes having a much longer shelf life and being less dangerous to handle. The total elimination of radioactivity from the probe is, of course, much more preferable. A possible way is to tag the DNA probe with biotinylated nucleotides (143).

PLASMIDS

Reports on extrachromosomal DNA in *A. laidlawii* and in several *Mycoplasma* species were based mainly on demonstration by CsCl density gradient centrifugation (68, 294) or by electrophoresis in agarose gels (111) of DNA bands additional to the chromosomal DNA band. Electron microscopy revealed covalently closed circular duplex DNA structures in the extrachromosomal band of *M. hominis* (294). Since these rather preliminary observations were not followed in depth, identification of the extrachromosomal bands as plasmids remained uncertain. Much more work has been invested in studying extrachromosomal elements in spiroplasmas, simply because of their abundance in these organisms. Ranhand et al. (211) were the first to report the presence of multiple covalently closed duplex DNA molecules in 10 of 12 different spiroplasma strains. The extrachromosomal DNA was detected by ethidium bromide-CsCl gradient centrifugation of cell lysates followed by examination of the resulting bands by electron microscopy and agarose gel electrophoresis. Two to eight size classes per strain made up of DNA molecules of 1 to 26 MDa were detected. In some strains, the amount of extrachromosomal DNA reached a value of 12% of the total cellular DNA (211). Resembling other bacterial plasmids, spiroplasma plasmids appeared as three forms in agarose gel electrophoresis and electron microscopy: covalently closed supercoiled circular, linear, and open circular. Subsequent work in several laboratories has confirmed the wide occurrence and great variety of extrachromosomal DNAs in spiroplasmas (10, 13, 180–182; Nur et al., submitted for publication).

The distinction of plasmid DNA from RFs of viral DNA

constitutes a difficult problem, as spiroplasmas are most often infected by a variety of viruses (see Mycoplasma Viruses section). Ranhand et al. (211) argued that the presence and sizes of the circular extrachromosomal DNA molecules detected by them appeared unrelated to either carriage or active production of known spiroplasma viruses. However, the recent finding that the RFs of the rodlike SPV1 virus (Table 2) consist of circular superhelical duplex DNA and nicked forms of it, 8.5 kb in size (63), calls for great caution, as the size and structure of these RFs resemble those of some of the reported spiroplasma plasmids (13, 180; Nur et al., submitted). Physical maps may be of great help in distinguishing the plasmids from each other and from viral DNA. Thus, the restriction pattern of the 8-kb plasmid pM41 isolated from an *S. citri* strain by Mouches et al. (180, 182) is essentially identical to that of plasmid pIJ2000 isolated from another *S. citri* strain by Archer et al. (10). The 8-kb plasmid pRA1 isolated by us from *S. citri* R8A2 differs, however, in its restriction pattern from the above plasmids (Nur et al., J. Bacteriol., in press). Moreover, pRA1 was successfully cloned in *E. coli*, whereas the two other plasmids could not be cloned (13, 180–182). Nevertheless, differences in restriction patterns of plasmid DNA might not rule out the possibility of plasmid relatedness. Thus, the 8-kb plasmids detected in the corn-stunt and 277F spiroplasmas were not digested by *Hin*dIII and *Mbo*I, enzymes capable of digesting the 8-kb pM41 plasmid isolated from *S. citri*. However, ^{32}P-labeled nick-translated pM41 DNA hybridized with the 8-kb plasmids of the corn-stunt and 277F spiroplasmas, indicating their relatedness (180, 181). It can be assumed that differences in susceptibility to digestion by restriction endonucleases may reflect minor differences in DNA structure, such as base replacement or methylation within the plasmid DNA sequences recognized by the restriction enzyme.

A most interesting phenomenon was observed in experiments in which nick-translated spiroplasma plasmids were used as probes for testing the occurrence of these plasmids in a variety of spiroplasma species and strains. The *S. citri* plasmid pMH1 (7 kb), found as a free plasmid in *S. citri* MH, was cloned into pBR328, and the ^{32}P-labeled nick-translated recombinant plasmid was used as a probe in Southern blot hybridization tests with *Hin*dIII- and *Mbo*I-digested DNAs of other spiroplasma strains (180–182). Positive hybridization results were obtained with chromosomal DNA of two *S. citri* strains and of several spiroplasma strains not belonging to the *S. citri* species. These data were interpreted by Mouches et al. (180–182) to mean that DNA sequences corresponding to the entire pMH1 DNA were integrated into the chromosome. It should be stressed that free pMH1 was not detected in these strains. Integration of plasmid DNA into the chromosome appears to be a phenomenon of wide occurrence in spiroplasmas. Our recent hybridization studies (Nur et al., submitted) using the nick-translated cloned 8-kb plasmid (pRA1) of *S. citri* as a probe show that *S. citri* R8A2 (Maroc) subclones, propagated for various periods in different media, harbor the free plasmid in quantities estimated to reach over 10% of the total DNA of the organism, or a combination of free plasmid and plasmid DNA integrated into the chromosome, or plasmid DNA integrated into the chromosome with very little or no free plasmid (Fig. 4). Chromosomal DNA sequences hybridizing with plasmid DNA were found to be abundant in all the spiroplasmas of serogroup I tested by us and to variable degrees in spiroplasmas belonging to serogroups different from that of *S. citri*, such as *Spiroplasma* sp. strain PPS-1 (serogroup IV), although DNA of other spiroplasmas (such as *S. apis* B31) gave negative results.

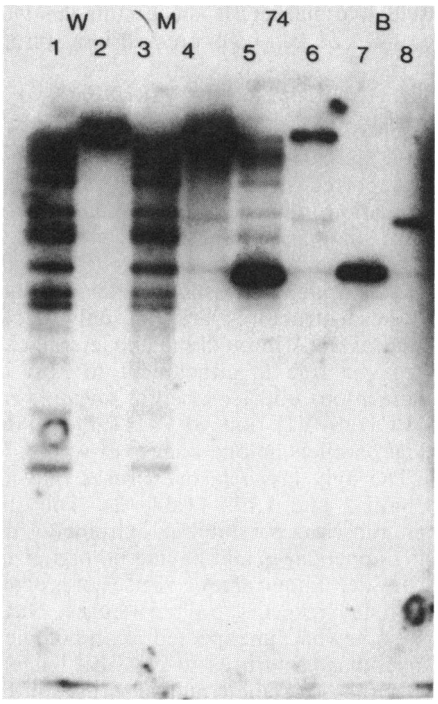

FIG. 4. Integration of the DNA of *S. citri* plasmid pRA1 into the chromosome. The nick-translated pRA1 plasmid was hybridized with *Hin*dIII-digested DNA of the *S. citri* R8A2 (Maroc) subclones W, M, 74, and B. The subclones differed in the number of passages, with B having the lowest and W the highest number of in vitro passages. Lanes: 1, digested DNA of W; 2, undigested DNA of W; 3, digested DNA of M; 4, undigested DNA of M; 5, digested DNA of 74; 6, undigested DNA of 74; 7, digested DNA of B; 8, undigested DNA of B. The pRA1 plasmid (8 kbp) has a single *Hin*dIII site and can be seen as a heavy band in lanes 5 and 7. The figure shows that the amount of free plasmid is highest in B, while the amount of plasmid DNA integrated into the chromosome is highest in subclones W and M (I. Nur, G. Glaser, and S. Razin, unpublished data).

The implications of plasmid DNA integration into the spiroplasma chromosome are not known as yet. The apparently considerable amounts of plasmid DNA integrated leave little doubt about its possible impact on gene expression in these spiroplasmas. Another aspect to be considered in this context is the effect plasmid DNA integration may have on chromosomal DNA-DNA hybridization data, commonly used in spiroplasma classification.

Regrettably, spiroplasma plasmids must be considered as cryptic plasmids, since no phenotypic traits could be attributed to them thus far. The notions that the plasmids are associated with pathogenicity, drug resistance, etc., remain unsubstantiated. One-dimensional or 2D-PAGE of *S. citri* strains harboring or lacking the plasmid pM41 failed to show any significant differences in cell proteins (182). The successful construction of hybrid plasmids constructed of *E. coli* plasmids with inserts of spiroplasma plasmid DNA may enable us to exploit techniques for transcription and translation of cloned DNA in *E. coli* in the search for the spiroplasma plasmid gene products. The hybrid plasmids may also be used in the opposite direction, by trying to transform with them spiroplasma cells. The chloramphenicol resistance gene of the chimeric plasmid may in this case be

used as a selective marker. If successful, this would mean that a gene of *E. coli* can be expressed in a spiroplasma.

MYCOPLASMA VIRUSES

The number of characterized mycoplasma viruses has been steadily increasing since 1970, the year in which Gourlay (92) reported the discovery of the first mycoplasma virus. Classification, nomenclature, and basic properties of the viruses are summarized in Table 2.

Many mycoplasma viruses resemble classical bacteriophages in having appendages such as tails and fibers, indicating that these structures are not only necessary for injecting the viral DNA through the eubacterial cell wall but apparently play a role in attachment to host cells. The nontailed filamentous and icosahedral mycoplasma viruses such as MVL1, SPV1, and SPV4 (Table 2) have their morphological parallels among phages of wall-covered bacteria (168). The only mycoplasma viruses that appear different are MVL2 and L172 (Table 2). These spherical, membrane-bound viruses exhibit an extremely simple shape devoid of any appendages and having no organized internal structure. They are unique among viruses of procaryotes and resemble, in many respects, animal viruses. Nevertheless, despite the somewhat unexpected morphological resemblance of mycoplasma viruses to classical bacteriophages, mycoplasma viruses resemble animal viruses in their mode of release from host cell. In many cases, mycoplasma virus release takes place by budding through the mycoplasma cell membrane, a continuous process not associated with cell lysis.

Several reviews on mycoplasma viruses were published in the 1970s. The most extensive ones were those by Maniloff et al. (167) and by Cole (43). More recent information on classification and characteristics of mycoplasma viruses can be found in the report of a Study Group on Phages of Mycoplasmas and Spiroplasmas (168). Techniques for propagating and characterizing mycoplasma viruses are detailed in *Methods in Mycoplasmology* (280).

Host Specificity and Virus Heterogeneity

Soon after the discovery by Gourlay (92) of the first rodlike virus in *A. laidlawii*, Liss and Maniloff (154) reported the isolation of similar viruses from several *Mycoplasma* species. However, the morphology and other properties of these viruses very much resembled those of MVL1, the rodlike virus of *A. laidlawii*. Furthermore, the viruses could only propagate on *A. laidlawii* lawns and not on lawns of the *Mycoplasma* species considered to be their original hosts (154). This apparent lack of host specificity stirred a controversy. Gourlay (94) raised doubts about the interpretation of the findings of Liss and Maniloff, arguing that these viruses were really *A. laidlawii* viruses, produced through the appearance of spontaneous plaques on lawns of the indicator strain or by activation of latent phages by the filtered material of the *Mycoplasma* cultures added to the *A. laidlawii* lawns. Experimental support for Gourlay's view was provided by Clyde (42), showing that two rodlike viruses obtained by inoculating *A. laidlawii* lawns with *M. gallisepticum* culture filtrates could not be distinguished serologically from the original MVL1 by cross-neutralization tests. Moreover, the viruses could not even adsorb to *M. gallisepticum*.

A consensus appears to have been reached now that use of *A. laidlawii* lawns for isolating viruses from *Mycoplasma* species is inadvisable. Thus, the recent isolation of a virus from *M. hominis* type 2 (*Mycoplasma arthritidis*?) on *A. laidlawii* lawns by Jansson et al. (126) is open to the above criticism. In fact, the virus isolated resembled the spherical *A. laidlawii* virus MVL2, leaving little doubt about its real origin. Nevertheless, as can be seen in Table 2, it has recently been established by Gourlay et al. (97, 99) that *Mycoplasma* species can harbor their own specific viruses. The viruses isolated were definitely different from *A. laidlawii* viruses and could not propagate on *A. laidlawii* lawns. Congdon et al. (46) reported, however, that MVL3, an established *A. laidlawii* virus, could also be propagated on lawns of *Acholeplasma modicum* and *Acholeplasma oculi*. Similarly, spiroplasma viruses such as SPV1 and SPV3 appear to infect a variety of *Spiroplasma* species. We may thus conclude that with regard to infectivity some of the mycoplasma viruses may cross the species barrier, but so far there are no examples of mycoplasma viruses crossing the genus barrier.

The phenomenon of multiple infection should be mentioned here. Some *A. laidlawii* and *S. citri* strains harbor more than one virus type simultaneously (43, 94). This may cause serious problems in mycoplasma virus studies. Thus, a strain of *A. laidlawii*, JA1, used in our laboratory for years to propagate MVL2 started to yield MVL3, probably as a result of changes in growth conditions (N. Greenberg and S. Rottem, personal communication). The possibility that the JA1 culture used by Greenberg and Rottem consisted of two cell populations, each carrying a different virus, should be considered. Accordingly, changes in growth conditions could have favored one of the cell populations. In Maniloff's hands, (personal communication) his established host strains, such as *A. laidlawii* JA1 and K2, yield consistently homogenous virus preparations. Yet Liss et al. claim in a recent paper (152) that their data support the proposal that one acholeplasma cell can replicate two different viruses at one time.

As could be expected, heterogeneity expressed by differences in virus composition, antigenicity, sensitivity to inactivating agents, and host specificity has been detected within the characterized mycoplasma virus types. Heterogeneity has been demonstrated among the rod-shaped *A. laidlawii* viruses, the so-called L1 (A1) group (154), the polyhedric L3 (A3) viruses (95), and among the S3 (C3) spiroplasma viruses (63–65, 278). For a long time the enveloped *A. laidlawii* virus MV-Lg-pS2-L172 (designated L172, Table 2) was considered to be closely related to MVL2, although comparative studies revealed some differences in composition and biological properties between those two viruses (4, 67, 98). Very recently Dybvig et al. (70), upon reexamination of L172, found that this virus differs from MVL2 in having a totally different genome structure and protein composition (Table 2), leading them to propose that L172 represents a new group of viruses.

Morphology

Mycoplasma viruses exhibit several major morphological entities (Table 2): rod-shaped naked viruses (MVL1 and SPV1), spherical viruses (MVL2, L-172, SPV4), and a variety of tailed viruses, differentiated according to their head structure, length of tail, and presence of a base plate, collar, fibers, etc. Illustrations and detailed morphological descriptions of mycoplasma viruses can be found in Cole's contributions (43, 44).

The morphological resemblance of MVL1 and SPV1 and their sharing of several other properties, including mode of

infection, turbid plaques, resistance to nonionic detergents, ether, and heating at 60°C suggested their possible relatedness. However, the finding that SPV1 is much longer than MVL1, produces larger plaques, differs serologically, and cannot infect *A. laidlawii*, while MVL1 cannot infect spiroplasmas, led Liss and Cole (150) to conclude that the two viruses, or virus groups, are not closely related, if related at all. The recent demonstration by Dickinson and Townsend (63) that the single-stranded circular genome of SPV1 is almost twice the size of that of MVL1 (Table 2) clearly distinguishes the two viruses. On the other hand, the basic structural similarity of the genome and RFs of SPV1 and MVL1 (63) support the inclusion of these viruses in the same family (168).

As mentioned above, MVL2 and L172 exhibit the simplest morphology among mycoplasma viruses. They are bounded by a single lipid-containing membrane and have no morphologically recognizable capsid structure (70, 200). In addition, solubilization of MVL2 by Triton X-100 excluded the possibility that any of the major polypeptides of this virus are associated with a capsid structure (247). In the words of Nowak and Maniloff (200), the MVL2 virion is a condensation of nucleoprotein bounded by a unit membrane. The superhelicity of MVL2 DNA may be instrumental in packing of the viral chromosome and in the assembly of the virion in the absence of a capsid structure. On the whole, relatively few viruses are known to contain superhelical DNA (200). Interestingly, MVL2 progeny were recently shown (204) to consist of three morphological quasispherical forms differing in diameter (about 70, 90, and 130 nm) but having the same genome, protein, and surface charge density. However, the virions of the intermediate size were found to carry two to three genomic copies, whereas virions of the other two classes contained only one genome.

Thus far, MVL2 and L172 appear to be the only well-established enveloped mycoplasma viruses. The spherical virus found to infect *A. modicum* (Table 2) is probably covered by an envelope, as it was sensitive to Nonidet P-40. However, this virus has been poorly characterized, so that definite conclusions as to its morphology, physicochemical properties, and infectious cycle must await further studies.

Among the tailed mycoplasma viruses some, like SPV2, have long tails and morphologically resemble the type B1 bacteriophages, while MVBr1 resembles the A1 phages. The short-tailed SPV3 and MVL3 viruses resemble the T7 phage group (168). It is of interest to mention that MVL3 and SPV3, when released from the host cells by budding through the cell membrane (see Virus Replication section) may be temporarily enclosed in membrane vesicles (43, 104, 168, 278). As in the case of MVL1 and SPV1, the MVL3 and SPV3 viruses show some resemblance in morphology, type of DNA, mode of replication, and release from cells. However, there can be no doubt that these viruses are different. Thus, the SPV3 virions are definitely smaller than the MVL3 virions (63–65, 278) and their host specificity is different (Table 2).

Biophysical and Biochemical Properties

Nucleic acids. The genome of all the mycoplasma viruses characterized so far is made of DNA. It occurs either as circular single-stranded DNA (MVL1, L172, SPV1, SPV4), linear double-stranded DNA (MVL3 and SPV3), or circular double-stranded DNA (MVL2). The genome of MVL1 (4,520 ± 380 nucleotides) is significantly smaller than the genome of other single-stranded DNA phages (201), making it a suitable candidate for sequencing. A physical map of the MVL2 genome constructed by Nowak and Maniloff (200) has been confirmed and extended by Dybvig et al. (71) and Honigman et al. (116). The G+C content of the MVL2 genome is 32 mol% (71) whereas that of L172 is 29 mol% (70). Both viral DNAs thus have a similar G+C content to that of the host cell DNA (31 mol%) (69). The genome of SPV3 contains 27 mol% G+C (unpublished observations of R. M. Cole cited in reference 168), and a physical map of one of the viruses of the S3 group (designed *ai*) has been recently constructed (64, 65). This virus has a 16-kbp genome of double-stranded DNA that can circularize due to cohesive ends. The nature of the nucleic acid remains unknown for some of the viruses infecting mollicutes (Table 2).

Proteins. MVL51 contains four structural proteins which apparently constitute the helically arranged subunits of the cylinder enclosing the viral DNA (168). Two other virus-specific proteins (10 and 14 kDa) have been detected in infected host cells and apparently play an important role in viral replication, as will be discussed below (57). The four structural proteins account for about 75% of the coding capacity of the MVL51 genome, suggesting the existence of more, still uncharacterized, virus-specific proteins.

The enveloped MVL2 virus shows four major proteins and five to six minor ones (Table 2). Although the published molecular weights of the major proteins show some variance, probably due to experimental and strain differences, the electrophoretic patterns are basically similar in showing a triplet of high-molecular-size proteins in the range of 60 to 80 kDa and another major protein at 14 to 19 kDa (98, 100, 157, 158, 207). None of the viral proteins gave a positive periodic acid-Schiff reaction, suggesting the absence of glycoproteins (247). Lactoperoxidase-mediated iodination of the virus particles revealed that at least two of the major proteins are exposed on the external virus surface, in accordance with their presumed role in virus attachment (247). An internal protein (17 kDa) not labeled by lactoperoxidase and resistant to proteolytic treatment of the virions may be involved in packaging of the viral DNA (Greenberg and Rottem, personal communication).

As to proteins of MVL3, Garwes et al. (83) reported five proteins (Table 2). However, unpublished data of K. Haberer (cited in reference 168) are at variance with those of Garwes et al. (83) by showing the presence of 10 viral proteins ranging from 15 to 59 kDa, in addition to a number of other virus-specific proteins found in the infected host cells.

Little is known about proteins of spiroplasma viruses. SPV3 contains five to seven proteins (Table 2) (45, 278). The published electrophoretic patterns show differences that can be attributed to heterogeneity of this group of viruses, reflected also in their biological properties (64, 65, 278). The newly discovered spiroplasma virus SPV4 contains a single major protein (of about 60 kDa) probably forming a capsid, but the presence of minor viral proteins cannot be excluded (235, 236).

Lipids. Morphological evidence for release of MVL2 by budding suggested that lipids of this virus, like those of enveloped animal viruses, are derived from the host-cell membrane (218). This prediction proved to be correct when Greenberg and Rottem (100) and later Al-Shammari and Smith (4) showed that the lipid species of MVL2 are the same as those of *A. laidlawii*. The lipid content of MVL2 was estimated to be 10 to 12% of the total dry weight of the virus, about the same as the values for bacteriophages PM2 and φ6 but lower than the values recorded for enveloped animal

viruses (100, 146). Electron-paramagnetic resonance spectroscopy of spin-labeled fatty acids incorporated into MVL2 indicated that the lipid domain of the virus envelope has the properties of a bilayer. The hydrocarbon chains in the MVL2 envelope were, however, less mobile than in the host-cell membrane, a difference attributed to the different content, composition, and disposition of proteins in the two membranes (100, 247). Differential-scanning calorimetry measurements by Putzrath et al. (207) confirmed the conclusions derived from the electron-paramagnetic resonance studies as to the bilayer nature of the viral lipid domain but indicated that the viral envelope was somewhat more fluid than that of the host. In view of the different experimental approaches, the electron-paramagnetic resonance and differential-scanning calorimetry data may not be mutually exclusive.

Since the MVL2 lipid composition is dictated by that of its host, it is possible to introduce controlled alterations in the fatty acid composition and in cholesterol content of the viral membrane simply by changing the fatty acid and cholesterol supplements of the growth medium employed for cultivating the host (4, 100, 207, 263). It became possible in this way to analyze the effects of changes in the physical state of both host and virus membranes on parameters such as virus adsorption, DNA penetration, and virus release (see relevant sections).

Virus Inactivation

Sensitivity of mycoplasma viruses to solvents, proteolytic and nucleolytic enzymes, antiviral antibodies, heat, and UV irradiation have served as effective means for differentiating strains within and among virus groups and as indicators for the presence of a lipid-containing envelope (54, 93, 167). Thus, inactivation of MVL2 by detergents and by diethyl ether was the first indication for a lipid-containing envelope in this virus (93). All the other mycoplasma viruses examined so far, apart from L172 (70) and the poorly characterized MVM1 isolated from *A. modicum* (Table 2), are resistant to detergents and diethyl ether.

Differences in UV inactivation curves were used by Das et al. (60) to differentiate among group L1 *A. laidlawii* viruses. Interestingly, the group L2 viruses were significantly less sensitive to UV inactivation than L1 viruses were. Part of this decreased sensitivity could be attributed to host-cell reactivation of the double-stranded DNA of group L2 viruses by the excision repair system of the host *A. laidlawii* cells (59, 60) a mechanism ineffective on single-stranded DNA.

Virus Adsorption

This initial stage in virus infection is of particular interest since, like animal viruses, mycoplasma viruses bind directly to the host plasma membrane, whereas the classical bacteriophages bind to the bacterial cell wall, flagella, or pili. The lipoglycan of the *A. laidlawii* membrane appears to serve as a specific receptor for MVL2. An *A. laidlawii* strain devoid of lipoglycan was incapable of adsorbing the virus (3, 5). Lipoglycans are composed of relatively long oligosaccharide chains linked to a diglyceride. The oligosaccharide chain acts as the active moiety in virus adsorption, since deacylation of the lipoglycan resulted in pure oligosaccharide which was more efficient in binding MVL2 than intact lipoglycan or isolated membranes. Receptor specificity is apparently determined by the oligosaccharide moiety. Thus, *A. oculi* lipoglycan did not bind the virus. The finding that protease treatment of *A. laidlawii* membranes decreased MVL2 adsorption suggests that *A. laidlawii* membrane proteins serve also as receptors for MVL2, in addition to lipoglycans (5). Decreased virus adsorption after proteolytic treatment of the host-cell membrane was also noticed for MVL1 adsorption (5, 55) but was most pronounced with MVL3, indicating that *A. laidlawii* membrane proteins serve as major receptors for this virus (5, 106).

Very little is known about the nature of viral components responsible for attachment to host-cell receptors. Two viral polypeptides exposed on the outer surface of MVL2 may play a role in recognition and attachment of this virus to *A. laidlawii*, since trypsin treatment of the virus markedly reduced its infectivity (247, 248). Electron microscopy of adsorbed MVL1 and MVL3 indicates that viral components responsible for attachment to *A. laidlawii* are apparently concentrated at one pole of the rodlike MVL1 and in the short tail of MVL3 (94, 105), but no information on their chemical nature is available.

Adsorption of the three *A. laidlawii* viruses (MVL51, MVL2, and MVL3) and of spiroplasma virus SPV3 (*ai*) followed first-order kinetics. Being dependent on pH and divalent cations, adsorption appears to involve electrostatic bonds (65, 103, 106). Up to 350 MVL3 particles were shown to adsorb to a single *A. laidlawii* cell. The adsorbed particles formed clusters at 37°C but were randomly distributed at 4°C; redistribution to clusters occurred when the temperature was raised to 37°C (95). This phenomenon was extensively studied by Haberer et al. (102, 103, 105, 107–109). They considered it to represent an active process of capping, caused by lateral diffusion of receptor sites on the *A. laidlawii* membrane surface, in a mode reminiscent of receptor clustering on eucaryotic cells by lectins or antibodies. Their finding that energy-blocking agents including dicyclohexylcarbodiimide and carbonyl cyanide *m*-chlorophenylhydrazone inhibited MVL3 clustering was taken in support of an active process. The mechanism of virus clustering is far from being clear. It could not be followed by light microscopy in living nonfixed preparations due to the minute size of the mycoplasmas and the viruses. A cytoskeleton resembling that responsible for capping in eucaryotic cells has not been described in *A. laidlawii*, although a primitive version of a cytoskeleton may be present in some mycoplasmas (for references see reference 195). The possibility that MVL3 clustering is driven by changes in *A. laidlawii* membrane fluidity induced by virus adsorption was not supported by fluorescence polarization measurements (109). Nevertheless, Haberer et al. (109) proposed that MVL3 capping is due to phase separation of membrane components. Accordingly, MVL3 by cross-linking to cell membrane proteins causes a phase separation, resulting in the formation of fluid patches of lipid regions with low protein content. The finding that *A. laidlawii* cells infected by MVL3 exhibit long filamentous appendages (108) and undergo fusion to produce giant cells may support the presence of fluid lipid patches (102). However, giant cells have also been observed in *A. laidlawii* infected with MVL1 and MVL2 (29).

The ability to introduce controlled alterations in fatty acid composition and cholesterol content of *A. laidlawii* membranes and consequently in MVL2 envelope lipids facilitated the analysis of effects of changes in membrane fluidity of both host and virus on virus adsorption and infectivity. Putzrath et al. (207) showed that near the lower limit of the *A. laidlawii* membrane lipid phase transition, cell growth was retarded, with no effect on virus adsorption or matura-

tion. More recently, Steinick and Wieslander (263) noticed that MVL2 adsorption rates were dependent on the acyl chain composition of the membranes, although no apparent correlation between lipid composition and plaque-forming ability was discernible. On the whole, it seems that significant alterations in membrane lipid composition of both host and virus failed to yield any dramatic changes in virus adsorption, penetration, and release. As the experiments must have been carried out on *A. laidlawii* cells in which at least part of the lipids were in a fluid state to enable cell growth (219), the lack of dramatic changes in the parameters tested could perhaps be expected.

Resistance to Virus Infection

Many *A. laidlawii* strains are resistant or immune to infection by the viruses known to infect this species. However, uninfected but susceptible cells can be found in infected cultures (97, 243). The question of how these organisms escape infection remains open. Susceptibility may depend on the physiological state of the organism, a parameter known to differ markedly among members of nonsynchronized microbial cultures.

The simplest explanation for resistance to virus infection is, perhaps, that based on the lack of receptors for virus binding. Thus, resistance of a JA1 strain of *A. laidlawii* to MVL2 could be attributed to the lack of lipoglycan (3). Susceptibility to MVL51 infection could be correlated with differences in membrane lipid composition among susceptible and resistant *A. laidlawii* strains (264). In the case of MVL2, resistance to infection may depend on changes in the envelope lipids of the virus itself, induced by its propagation in different hosts (153), but the studies of Rottem and Greenberg (248) point to the dominant role of MVL2 proteins in adsorption. Still, it can be argued that changes in viral lipids and consequently in viral membrane fluidity may affect virus adsorption by influencing the degree of exposure of viral proteins on its surface (219).

Restriction and modification of viral DNA by host-cell methylases may provide another explanation for host resistance. Maniloff and Das (166) were the first to report on host modification and restriction of MVL2. The biochemical basis of this phenomenon was later elucidated by Dybvig et al. (71), who analyzed methylated bases in the DNA of two *A. laidlawii* strains (K2 and JA1) and in the DNA of MVL2 propagated in these hosts. The virus grown on JA1 had a plating efficiency of 0.05 on strain K2 relative to its plating efficiency on strain JA1, while the plating efficiency of the virus grown on K2 was only 0.002 on JA1 relative to that on K2. The DNA of the virus grown on K2 contained m^5Cyt and m^6Ade, resembling the DNA of the host. Clearly, in this case a restriction modification based on DNA methylation is operative. However, both the DNAs of JA1 and the virus grown in it had no detectable methylated bases. Dybvig et al. (71) proposed, therefore, that JA1 may modify the viral coat rather than the viral DNA. However, the finding by Sladek and Maniloff (258) of similar restriction frequencies for infection of JA1 with intact MVL2 virus grown on K2 and transfection with DNA of the same virus supports the conclusion that modification and restriction in JA1 is also mediated at the DNA level. Very similar data leading to the same conclusions were recently reported for the other enveloped *A. laidlawii* virus, L172 (70). It should be mentioned that persistent nonlytic infection of *A. laidlawii* by MVL2 is common and is characterized by resistance to superinfection by the homologous virus. However, as expected from a lysogenic state, the mycoplasmas retain the potential of producing MVL2 when induced by a variety of agents such as mitomycin C and UV light (209).

Virus Replication and Release

The lack of a cell wall in mycoplasmas should facilitate viral DNA penetration and, in fact, transfection of mycoplasmas with purified DNA of several mycoplasma viruses has been achieved (see Transfection section). It is no surprise that in general we cannot find in mycoplasma viruses the elaborate injection mechanism reported for many bacteriophages. The tails of MVL3, SPV2, and SPV3 (Table 2), although reminiscent of bacteriophage tails, show no evidence for contractibility (94). The recently-described MV-Br1 appears to have a contractile tail (97). Nevertheless, the fact that MVL3 attaches to the mycoplasma cell through its tail led Gourlay et al. (95) to suggest that this virus injects its nucleic acid in a manner similar to that of tailed bacteriophages. The rod-shaped MVL1 and SPV1, the spherical SPV4, and the enveloped MVL2 and L172 have no tails at all. The finding of hollow rods of MVL1 attached to *A. laidlawii* cells has suggested that viral DNA is released into the cell after the tip of the virus is broken after attachment to the cell (94). It is plausible that the DNA of MVL2 gets into the host cell after fusion of the viral envelope with the cell membrane (263), but no experimental support for this supposition is available.

The molecular events of MVL51 DNA replication inside the host cell have been extensively studied by Maniloff and his colleagues and were described in detail in previous reviews (167, 218). In short, the parental single-stranded viral DNA is first converted to double-stranded RFs made up of covalently closed double-stranded molecules (RFI) and relaxed derivatives (RFII). The conversion of parental DNA to the RF probably occurs during penetration, as parental RF DNA is mostly membrane associated. Synthesis of progeny RF DNA is by symmetric replication at sites on the cell membrane. Synthesis of progeny single-stranded DNA occurs by asymmetric replication, most probably on cytoplasmic RF molecules. Virus maturation presumably occurs at the cell membrane as the virion is assembled and extruded. Similar RFs have recently been described for the rod-shaped SPV1 (*aa*) virus infecting *S. citri*, suggesting that this virus replicates by a mechanism resembling that described for MVL51 (63).

The scheme of MVL51 replication has been recently supplemented with several details concerning the role of virus-specific proteins in replication (57, 58). Chloramphenicol did not interfere with virus adsorption and DNA penetration. Moreover, it did not affect the conversion of parental single-stranded DNA to parental RF molecules, indicating that these early steps in virus replication do not require a virus-coded protein or synthesis of cell protein. However, the replication of RF molecules was completely inhibited by the antibiotic, apparently by inhibition of the synthesis of a 14-kDa virus-specific protein. Chloramphenicol also inhibits at a later step, that of the synthesis of progeny single-stranded DNA, apparently depending on the synthesis of another protein of 10 kDa (57, 58). A variant of *A. laidlawii*, REP^-, defective in replication of parental RF to progeny RF, was isolated by Nowak et al. (199). Recently, Sladek and Maniloff (259) found, to their surprise, that REP^- cells can be transfected with MVL51 DNA and produce viruses (see Transfection section). They propose that *A. laidlawii*

carries a transmembrane protein (REP) that functions in viral adsorption and penetration and also anchors viral DNA to membrane sites at which parental RF is replicated to progeny RF molecules. In the REP$^-$ variant this protein is altered, impairing its function. In transfection, since entry of DNA does not require viral adsorption, the protein is bypassed. Viral DNA, in this case, may attach to membrane sites other than REP or alternatively replicate in the cytoplasm.

The replication of the superhelical double-stranded DNA of MVL2 apparently follows a pattern resembling that of the bacterial chromosome. Novobiocin inhibits MVL2 replication, probably through its known inhibitory effect on DNA gyrase activity required to transform the superhelical viral DNA into open circular double-stranded DNA (205). Podder and Maniloff (205) suggest that in addition to inhibiting gyrase activity, novobiocin inhibits some undefined step(s) in the process of virus assembly and release. As to MVL3, its DNA replication was found to involve intermediates sedimenting faster than viral DNA. However, Haberer and Maniloff (104) experienced difficulties in characterizing the MVL3 replicative intermediates, since viral DNA could not be effectively separated from cellular DNA. There was a significant decrease in DNA synthesis in *A. laidlawii* cells during the first hour after MVL3 infection. During this period about 40% of the nascent DNA was viral. After 2 h of infection, 60 to 80% of the DNA was viral, and by the end of 3 h all nascent DNA was viral (104). Although MVL3 resembles coliphage T7 in morphology and chromosome size, the replication of MVL3 DNA inside the host was not associated with breakdown and reutilization of host-cell DNA, as reported for T7. Moreover, while T7 is released in a burst at about 25 min after infection at 37°C, MVL3 virus production continues for hours by the biosynthetic apparatus of the infected cells (104). Clearly, the fact that MVL3 replication does not induce cell lysis enables this prolonged process of virus production.

With the possible exception of the newly discovered spiroplasma virus SPV4, none of the viruses infecting mycoplasmas has been shown to be lytic in the sense that infectious virus particles accumulate until they are released by lysis of the host cell. Virus release from mycoplasmas is usually gradual and continues for hours, resembling the release of viruses from animal cells. The total number of virions released ranges from a few dozen to about 300 per mycoplasma cell (167, 218). It appears that the rod-shaped MVL1 and SPV1 (278) assemble as they emerge through the cell membrane, as no intracellular structures are apparent in infected cells (151). Thin sections suggest that the enveloped MVL2 virus is released by budding through the host-cell membrane. After a latent period of about 90 to 120 min, there was a continuous release of progeny MVL2 virus for about 4 to 6 h, without causing cell lysis or death (165, 205). The picture appears somewhat different with MVL3. In this case, virus particles have been observed in the cells before their release (96). The particles align radially with their tails oriented toward the inner face of the cell membrane (107). The MVL3 virions, as well as the morphologically similar SPV3 and spv-1 viruses, are then released either singly or in small groups temporarily contained within membrane-bound vesicles. These vesicles are most probably formed by budding through the host-cell membrane, but how they break down to release naked virus particles is not clear (43, 103, 106, 278, 288). MVL3 release does not cause cell lysis; yet the organisms are killed. This may explain the clear nature of MVL3 plaques. Plaques of MVL1 and MVL2 are turbid, signifying the viability and slow growth of the infected *A. laidlawii* cells (167). The recently described spiroplasma virus SPV4 (Table 2) resembles SPV3 in causing a cytocidal infection. However, there is no evidence for the release of this virus by budding. The SPV4 virions appear rather to accumulate inside the host cells before being released by cell lysis (235, 236).

Lysogeny

The early findings that infection of *A. laidlawii* with MVL1 and MVL2 is nonlytic and produces turbid plaques pointed to the possibility of a virus carrier state or lysogeny. It is still unclear whether MVL1 infection in *A. laidlawii* can be regarded as lysogenic. Claims that MVL1 release from *A. laidlawii* is stimulated by UV irradiation and mitomycin C (149, 154), agents known to induce lysogeny, have not been taken as constituting firm proof for MVL1 lysogeny, mainly due to difficulties in reproducing the results. According to Liss (149), unsuccessful reproduction of results is attributed to the use of *A. laidlawii* cultures at the mid-logarithmic phase instead of the late-logarithmic or stationary phase in which virus induction by mitomycin C is most pronounced. There is general consensus, however, that MVL2 infection fulfills the criteria for lysogeny. Thus, the data of Putzrath and Maniloff (208, 209) show that MVL2 can establish a persistent infection resembling that described for noncytocidal enveloped animal viruses. Cells retain the potential of producing the virus as a stable heritable trait, and both mitomycin C and UV induction increase the number of infectious centers. Persistently infected clones are also resistant to superinfection by homologous virus.

The molecular events occurring during establishment of the lysogenic state with MVL2 have been recently elucidated by Dybvig and Maniloff (69). They applied nick-translated MVL2 DNA as a probe to detect MVL2 DNA sequences in lysogenic cells by DNA-DNA hybridization using the Southern blot technique. Digestion of a lysogenic clone DNA with *Xba*I showed that these cells contained MVL2 DNA integrated into their genomes. *Xba*I cuts MVL2 DNA into two pieces. The *Xba*I digests of the lysogenic cell DNA showed two bands containing MVL2 DNA but having together a significantly higher molecular weight than the two bands of *Xba*I-cleaved linear MVL2 DNA. Dybvig and Maniloff (69) concluded that the lysogenized *A. laidlawii* cells contained a single MVL2 genome integrated into their chromosome. A single integration site was found in both viral and cellular DNA; no secondary integration sites could be detected.

Lysogeny has also been proved for SPV3 virus *ai* infecting *S. citri*. The lysogens were immune to superinfection by *ai* but not to infection by two serologically related viruses of the same group (64). As with MVL2, the SPV3 genome was shown by Southern blots to be integrated with the host-cell chromosome, employing as probes labeled *ai* DNA and cellular DNA digested by *Bgl*II, an enzyme which does not cleave the viral genome. The presence of high-molecular-weight DNA bands indicated integration (64). All the *S. citri* strains examined by Dickinson and Townsend (64) contained a deleted form of *ai* integrated as a cryptic prophage which was unable to confer resistance to *ai* superinfection. It appears that the *ai* genome had integrated at a unique point on the bacterial chromosome and subsequently lost the immunity region and one attachment site due to a deletion. The resultant cryptic prophage can thus no longer confer

immunity to superinfection and cannot be excised from the host genome. Nevertheless, the stable *ai* lysogens also contained a complete *ai* genome. Dickinson and Townsend (64) propose that the infecting viral DNA integrates adjacent to the cryptic prophage in a site-specific recombination event at unique points in both the virus and host genomes. Thus, lysogens also spontaneously release low levels of *ai* virus. The presence of virus DNA integrated into the *S. citri* chromosome is reminiscent of cryptic plasmid DNA integrated with the chromosome of the same spiroplasma (180; Nur et al., submitted). Hence, one must be aware of the possible occurrence of infective and cryptic viruses as well as plasmids in the same organism.

A peculiar plaque morphology of the *ai* virus, consisting of concentric rings differing in turbidity, resembles plaques produced by temperate bacteriophages (278). The explanation for this peculiar phenomenon is based on the finding that infection of bacteria at low multiplicities more often results in a lytic response, thus accounting for the clear center of the plaque, while at high multiplicities of infection lysogeny is the frequent outcome. So, as the concentration of virus in the plaque increases, cells will become lysogenized and consequently resistant to further infection. A few of these cells release low numbers of virus particles which cause a second zone of lysis (278).

Virus Infection and Mycoplasma Pathogenicity

It could be assumed that virus infection may affect mycoplasma pathogenicity, either by inducing genetic changes causing alterations in mycoplasma membrane antigens, production of toxic substances, or simply by killing the mycoplasmas in the animal or plant host. Evidence for virus-induced modifications of pathogenic factors associated with the mycoplasma cell itself is still unavailable. Nevertheless, MVL2 was shown to induce interferon production in sheep lymphocytes *in vitro* (157). The mechanism of interferon induction by MVL2 and the relevance of this finding to pathogenicity are unclear. More convincing evidence comes from recent studies with spiroplasmas. Alivizatos et al. (1) showed that transmission of *S. citri* containing the SPV3 virus *ai* to periwinkle plants already infected with a highly pathogenic *S. citri* strain resulted in suppression of symptoms and a reduction in the number of viable spiroplasmas. The virus could be seen to multiply in the spiroplasmas within the plant. The tendency of the *ai* virus to cause lysogenization (64, 65) should also be considered in this context. These viruses may establish a persistent infection of the spiroplasmas in the plant, slow the growth of the pathogen, and consequently cause amelioration of symptoms. Of relevance also is the finding that subsequent to the infection with the *ai* virus, spiroplasma cells infected with an SPV1-type and even with the rare SPV2-type virus were observed (278).

Another case in which virus infection may affect spiroplasma pathogenicity concerns the sex-ratio spiroplasma, which causes male sterility in *Drosophila* species. The spiroplasmas in the insect are frequently infected by viruses, which apparently belong to the SPV3 group (Table 2). Virus infection results in lysis of the sex-ratio spiroplasmas, and in this way the infection may be cured (167, 288). It has recently been proposed that the phenomenon of spiroplasma clumping brought about by mixing hemolymph from different *Drosophila* species is also associated with virus infection (203). It could be that clumping is the result of virus-induced changes in the spiroplasma membrane, or it could be simply the result of release of some of the viscous cellular DNA, causing the spiroplasmas to stick together.

PHYLOGENY

Models for Mycoplasma Phylogeny

The uncontested status of mycoplasmas as the smallest and simplest self-replicating organisms has put special weight on elucidation of their place in the global evolutionary scheme. Two contrasting models for mycoplasma evolution have been proposed. According to the first model, the mycoplasmas represent the descendants of organisms that preceded the procaryotic-eucaryotic cell split (179), or, in other words, the extant mycoplasmas are the surviving descendants of exceedingly primitive bacteria that existed before the development of a peptidoglycan-based cell wall. The second model claims that mycoplasmas represent degenerate eubacterial forms (194, 290) and can thus be considered as a product of a rather late development in evolution.

The absence of fossil records constitutes a major difficulty hampering construction of phylogenetic trees for bacteria. It is conceivable that even when taxonomic distance between mycoplasmas and other bacteria can be determined, this may not provide the direction of time. Consequently, construction of phylogenetic trees would not necessarily determine whether mycoplasmas predate or are derived from eubacteria (177). Although the controversy on the line of descent of mycoplasmas appears at the present to be of a more philosophical nature, it does have important taxonomical implications. Thus, supporters of the model based on evolution of mycoplasmas from eubacteria went as far as to suggest that mycoplasmas do not deserve a separate class status (290).

The first model for mycoplasma evolution, that proposed by Morowitz and Wallace (179) considers mycoplasmas to be the most primitive organisms existing today. While discussing the theoretical minimal cell, Morowitz (177) finds the mycoplasmas to be best fitted to this concept, as these organisms have the simplest structure, the smallest genome, and a minimum of metabolic pathways. According to Morowitz and Wallace (179), the mycoplasmas should be placed at the root of the phylogenetic tree. Evolution occurred through genome doubling, from the 500-MDa genome of *Mycoplasma* and *Ureaplasma* species to the 1,000-MDa genome of *Acholeplasma* and *Spiroplasma* species. Thus, acholeplasmas and spiroplasmas can be regarded as intermediates in the evolution from the small-genome mycoplasmas to the wall-covered procaryotes. The contestants of this model find it difficult to accept the variety of biochemical assumptions underlying it, such as considerable genome size increases and lengthening of the 5S rRNA molecule (see Ribosomal Ribonucleic Acid section). Moreover, the concept of genome size increases in progressing evolution stands in sharp contrast to the attractive, more recent hypothesis (66) that evolution moved in the direction of "streamlined" bacterial genomes with little noninformational DNA. Accordingly, mycoplasmas must be regarded as the furthest evolved of the procaryotes, since their genomes are the smallest and carry only the minimum number of genes essential for growth (164).

Moving on to discussing the second model, according to which mycoplasmas represent degenerate eubacterial forms, one can distinguish two variations of it. The first one,

promoted by Neimark (193, 194, 196), regards mycoplasmas as a broadly diverse assemblage of wall-less procarytes, made up of organisms that descended from various bacterial progenitors. The second variation, proposed by Woese et al. (290, 291), claims that mycoplasmas are clustered on a single phylogenetic branch arising deep within the gram-positive part of the eubacterial tree. This branch also contains two clostridia species, *C. innocuum* and *C. ramosum* (164, 244). For many years Neimark has concentrated his efforts on obtaining experimental support for his thesis that acholeplasmas evolved from streptococci. The variety of arguments brought up by Neimark in support of this thesis, including similar metabolic pathways and serologically related key enzymes, was recently summarized (197). However, comparison of the acholeplasma and streptococcus genomes (194, 196) leads to the conclusion that during the presumed transition of streptococci to acholeplasmas a third or more of the streptococcus genome, including at least three to four rRNA operons, was lost, so that even Neimark (194, 196) agrees that acholeplasmas are radically altered from streptococci. Those who object to Neimark's model also argue that it is not substantiated by macromolecular phylogenetic data and that it represents a far too limited view of the relationship of acholeplasmas to other mycoplasmas and to streptococci (244).

Ribosomal Ribonucleic Acids as Phylogenetic Markers

The first phylogenetic analysis of mollicutes by Woese et al. (290) was based on construction of 16S rRNA oligonucleotide catalogs, obtained by T1 ribonuclease digestion of 16S rRNA of several representative mycoplasmas. The catalogs included only hexamers and larger oligonucleotides, as only these are considered reliable indicators of molecular primary structure homology. Comparison of the mycoplasmal catalogs with those of other bacteria led Woese et al. (290) to conclude that mycoplasmas arose by degenerative evolution as a deep phylogenetic branch of the subline of clostridial ancestry that led to the genera *Bacillus*, *Lactobacillus*, and *Streptococcus*. Reliance on 16S rRNA oligonucleotide catalogs in mycoplasma phylogeny has met with some criticism. Thus, use of the catalogs suffers from the deficiency that a considerable portion of the 16S rRNA molecule yielding oligonucleotides smaller than hexamers is not taken into account (188). Availability of an increasing number of fully sequenced 16S rRNA molecules will enable, in the very near future, comparative analysis of entire molecules, thus eliminating the problem. Another, much more serious, difficulty was encountered by Woese et al. (290). A relatively high proportion of the highly conserved oligonucleotide sequences that commonly occur in every eubacterial 16S rRNA were not found in mycoplasmal 16S rRNAs. The mycoplasmal catalogs show a high proportion of unique sequences, differing among the various mycoplasmas. This tendency was much more pronounced in the mycoplasmas than in their presumed clostridial relatives (291).

To alleviate this difficulty, Woese et al. (290) compared mycoplasma catalogs with one another and with those of normal bacteria without considering the highly conserved sequences. This modification was considered by Neimark and London (197) as a possible fault that could have led to erroneous conclusions. To explain the occurrence of the unique oligonucleotides in mycoplasmas, Woese et al. (290, 291) suggest that mycoplasmas are in a state of rapid evolution, brought about by high mutation rates. The fact that the mycoplasma genome is only one-fourth to one-eighth the size of a typical bacterial genome will, theoretically, enable an abnormally high mutation rate. For example, even with a mutation rate three to six times higher than that of *E. coli*, a mycoplasma could still replicate its entire genome with the same overall accuracy. Clearly, by elevating mutation rates, a line of descent can accumulate a far more varied field of variants than does a normal line of descent. This would account for the rare and unique sequence variations in mycoplasmal rRNAs and for other bizarre properties, such as exclusive CpG methylation characterizing *Spiroplasma* sp. strain MQ-1 (Methylated bases section). Moreover, the presumed elevated mutation rate might account for the great genotypic and phenotypic variety of the organisms included in the class *Mollicutes* and would be in line with their wide occurrence and adaptability to different hosts and ecological niches. Furthermore, it would explain their relatively fast evolutionary branching from a defined ancestor (*C. innocuum* or *C. ramosum*), rejecting in this way Neimark's hypothesis that mycoplasmas had multiple origins, evolving from a variety of eubacteria.

Support for the hypothesis that mycoplasmas are descendants of gram-positive bacteria comes from recent sequencing data of mycoplasmal tRNAs and rRNAs. The structural features of the few mycoplasmal tRNAs sequenced so far resemble more those of the corresponding tRNAs of gram-positive than of gram-negative bacteria (133, 245, 283). Analysis of 5S rRNA sequence homology has proven to be a reasonably accurate method for the construction of phylogenetic trees of procaryotes and eucaryotes (118). The recent sequencing of 5S rRNA from 13 different *Mycoplasma*, *Ureaplasma*, *Anaeroplasma*, and *Spiroplasma* species, as well as *C. innocuum*, was used by Rogers et al. (244) to construct a phylogenetic tree which basically conforms with the ideas of Woese et al. (290). However, Rogers et al. (244) go further and propose a scheme for mycoplasma evolution (Fig. 5). Accordingly, the initial event in mycoplasma phylogeny was the formation of the *Acholeplasma* branch as an outgrowth of the *C. innocuum* branch of the low-G+C gram-positive eubacteria. This event must have involved significant chromosomal deletions to bring down the genome size to 1,000 MDa and was accompanied by loss of the cell wall. A splitting of this branch led to the sterol-requiring organisms ancestral to *Spiroplasma* species. Since oxygen is essential for sterol synthesis, it can be presumed that appearance of sterol-requiring mycoplasmas took place only after evolution of an oxidizing atmosphere on earth. The *Anaeroplasma* branch is probably close to the node between *Acholeplasma* and *Spiroplasma* branches. Quite surprisingly, there appears to have been repeated independent genome reductions, each to about 500 MDa, during evolution of the *Spiroplasma* branch to *Mycoplasma* and *Ureaplasma* species (Fig. 5). The biochemical and ultrastructural diversity of *Mycoplasma* and *Ureaplasma* species must reflect the multiple origins of these organisms, in accord with the idea that mollicutes represent a group in a state of rapid evolution.

The recent finding by Yamao et al. (292) that *M. capricolum* is using the nonsense or termination (opal) codon UGA as a tryptophan codon, a property shared only with mitochondrial genes, led to the suggestion that the presumed procaryotic progenitors of mitochondria could be mycoplasma-like organisms. This exciting prospect appears rather unlikely in light of the lack of quinones and cytochromes in mycoplasmas and the finding that

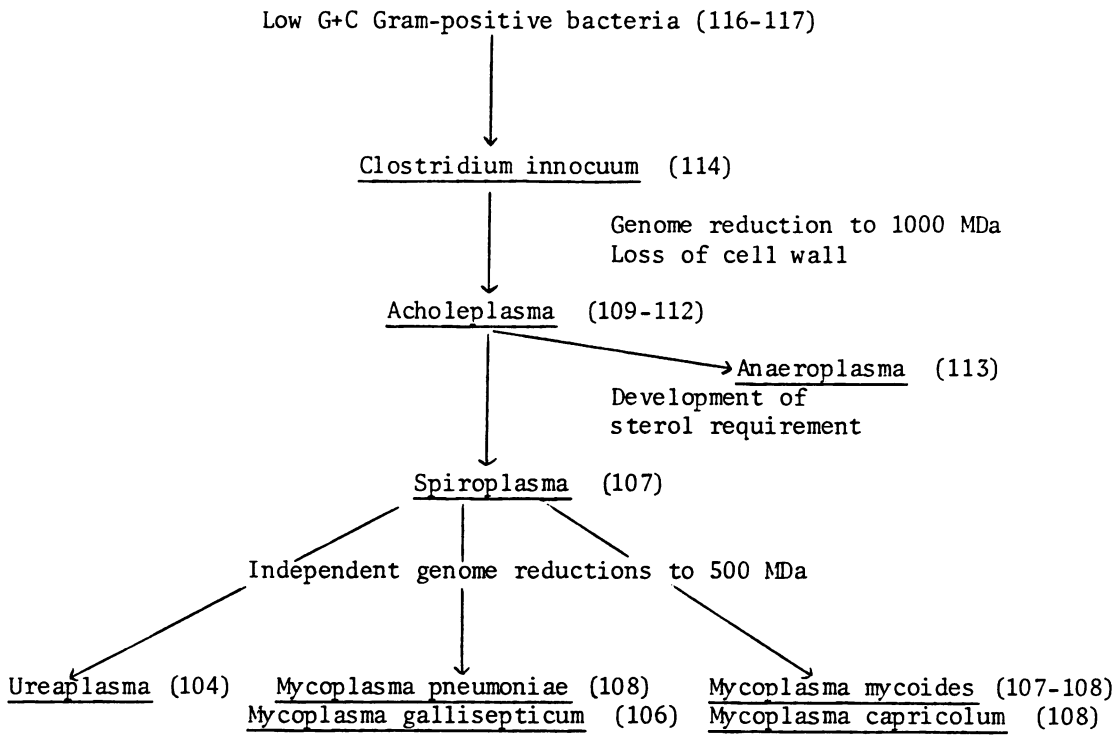

FIG. 5. Scheme for mycoplasma evolution according to Rogers et al. (244). Numbers in brackets denote number of nucleotides in 5S rRNA.

mycoplasmal rRNA genes show very little homology to the corresponding mitochondrial genes (see Ribosomal Ribonucleic Acid Genes section).

Relationship of Taxonomy to Phylogeny

The new ideas of Woese et al. (290) on mycoplasma phylogeny have rekindled the old controversy concerning the taxonomic status of mycoplasmas. The notion that mycoplasmas are stable L-forms of existing wall-covered bacteria was put to rest in the early 1960s after introduction of DNA hybridization techniques to mycoplasma classification. These tests revealed no genetic relatedness between any of the mycoplasmas and their presumed wall-covered parents (see reference 216). In retrospect, this controversy was more destructive than constructive, since it introduced a great deal of confusion and uncertainty as to mycoplasma classification and nomenclature. By putting an end to this dispute, the way was opened for establishing a proper and acceptable classification of the group. In 1967 an international group of mycoplasmologists, later to become the ICSB Subcommittee on Taxonomy of *Mycoplasmatales*, proposed to endow the mycoplasma group with a class status. The name chosen was *Mollicutes* (mollis, soft; cutes, skin), to denote the lack of a cell wall as a major distinguishing property (72). The provision of the high class status to mycoplasmas has been, in retrospect, an adequate and productive move, as it has helped to accommodate within this broad frame the many phenotypically and genotypically different mycoplasmas cultivated so far, taking also into account that the 100 or so established species represent only a portion, most probably a minor one, of the mycoplasmas existing in nature and uncultivated so far.

The significant advancement of our knowledge of the molecular biology and genetics of mycoplasmas achieved during the past decade and the introduction of new ideas and tools in phylogeny studies (76) require a reassessment of the taxonomic status of mycoplasmas. It is not surprising, although it is somewhat distressing to mycoplasmologists, to find statements which say that the "molecular biologists and biochemists working with mycoplasmas find their classification to have no phylogenetic sense" (133). Admittedly, the current classification of bacteria is based mostly on phenotypic characteristics, even though the most extensive phenotypic analysis tests only some 10 to 20% of the total genetic capacity of a bacterium. The significant gap between phenotypic and genetic classification is caused mainly by the ill-defined species concept in bacteria, although this taxonomic entity is still the basic unit on which classification is built. Definition of a bacterial genus is even more subjective, and the same can be said about higher taxa.

The proposal by Gibbons and Murray (86) to divide the procaryotes into four divisions according to the nature of their cell walls can be presently regarded as the best solution, as long as phenotypic characteristics govern bacterial classification. The four divisions, *Gracilicutes* (gram-negative bacteria), *Firmicutes* (gram-positive bacteria), *Tenericutes* (*Mollicutes*, the mycoplasmas with no cell walls), and *Mendosicutes* (the archaebacteria with modified cell walls) are easily distinguished by a variety of morphological and biochemical tests (188). Furthermore, since cell wall synthesis is dictated by a significant number of genes, classification on this basis also has some genetic relevance.

As any other classification system, it has its faults. The outstanding example for imperfection concerns the status of *T. acidophilum*. Lacking a cell wall, it was first included in *Mollicutes* (53) although with some hesitation due to its peculiar properties (77). However, the extensive investigations carried out on this thermoacidophile indicated without any doubt that it is an archaebacterium. Its 16S rRNA oligonucleotide catalogs (290), 5S rRNA sequence (160), tRNA properties (133), peculiar organization of rRNA genes (279), RNA polymerase properties (293), histone-like proteins (62), ether lipids (142) presence of quinones and cytochromes (115), lack of a proton-translocating adenosine triphosphatase (256), simple nutritional requirements (260), free-living nature (53) and flagellar motility (20) are all properties justifying the exclusion of this wall-less organism from *Mollicutes*. Hence, the lack of a cell wall by itself does not necessarily mean that the organism is a mycoplasma. From the point of view of those who believe in degenerative evolution, the finding of *T. acidophilum* suggests that loss of a cell wall arose at least twice, once during evolution of archaebacteria to produce *Thermoplasma* and at least once more during the evolution of gram-positive bacteria to produce the mycoplasmas (164). In practice, the isolated case of *T. acidophilum* does not cause a serious problem to the bacteriologist, since this organism can be easily distinguished from the mycoplasmas phenotypically and genotypically.

There is no real difficulty for a trained bacteriologist to distinguish mycoplasmas phenotypically. They form a stable and distinctive group. The criteria for definition of a new isolate as a mycoplasma have been clearly stated (268) and have been found to be effective. The only problem is to try and bridge the gap between the proponents of classification by phylogeny and the classical bacterial taxonomists. The statement by Woese et al. (290) that mycoplasmas do not constitute a phylogenetically coherent group and therefore do not deserve a distinct higher level taxon, as the name *Mollicutes* would suggest, has met with great opposition by the Subcommittee on Taxonomy of *Mollicutes* (269). The abolishment of mycoplasmas as a taxonomic group without providing an alternative would mean returning to the uncertainties and chaotic taxonomic situation existing at the peak of the L-form dispute. As Murray (188) puts it: "The practical bacteriologist needs a simple scheme of classification as a framework for recognition. At this stage adopting the ideas of Woese et al. will be confusing to the practical bench worker or the physician, and will thus be unhelpful." Clearly, every bacterial taxonomist will favor a taxonomic scheme based on genetic relatedness and hopes that there will come a time when we can set up taxa that are precisely defined in terms of molecular genetics. Unfortunately, this time has not yet arrived (188).

CONCLUSION

The mycoplasmas (mollicutes) are the smallest and simplest self-replicating procaryotes and are the closest to the concept of a minimal cell. They are widely distributed in nature as parasites in humans, vertebrates, plants, and arthropods. The 500-MDa (700-kbp) genome characterizing *Mycoplasma* and *Ureaplasma* species and the extremely low G+C content of it impose considerable restrictions on coding capacity, explaining the low number of cell proteins produced, the scarcity of metabolic pathways, and the complex nutritional requirements of mycoplasmas.

The mycoplasma genome resembles that of other procaryotes in containing m^6Ade or m^5Cyt or both. The finding that in *Spiroplasma* sp. strain MQ-1 only cytosine residues in the CpG doublet are methylated is of special interest, as this constitutes a typical eucaryotic trait. DNA methylation appears to play a role in restriction and modification in mollicutes as in other procaryotes. Whether methylation has other roles is still a moot point.

The scarcity of morphological and biochemical properties and the frequent occurrence of common antigens among mycoplasmas hampers their identification and classification. Introduction of new DNA-DNA hybridization techniques, Southern blots with specific gene probes, DNA cleavage patterns by restriction enzymes, and 2D cell protein maps have provided effective tools for determining genetic relatedness among strains and in this way solve taxonomic problems hard to solve by the conventional methods.

Information on mycoplasma genome replication is still fragmentary. The finding of only one DNA polymerase in *M. orale* and *M. hyorhinis*, lacking the 3′→5′ exonuclease activity, is another of the unique properties of mycoplasmas and raises the problem as to the proofreading mechanism in these organisms. Nevertheless, in several spiroplasmas three DNA polymerases were found, resembling the situation in *E. coli*. However, the question of whether these polymerases possess exonuclease activity has not been answered as yet. Photoreactivation and dark repair of UV irradiation damage were shown to operate in *A. laidlawii* but to be totally absent from *M. gallisepticum*, again raising the problem of repair and proofreading mechanisms in this organism. Is this finding associated with a higher mutation rate? The much smaller genome of mycoplasmas would theoretically allow their replication with the same overall accuracy is that of *E. coli*, even with a mutation rate three to six times as high (291). However, experimental data to support this supposition are not available.

The lack of a cell wall in mollicutes would seem to favor genetic exchange by transformation. Yet, despite many trials, only one study (80) succeeded in transferring tetracycline resistance, apparently through chromosomal transfer. The potent nuclease activities of mycoplasmas as well as their tendency to lose viability in buffer solutions may hinder transformation experiments. Transfection of mycoplasmas with mycoplasma viral DNAs has met with more success, particularly with the aid of polyethylene glycol. Successful transfection opens the way for the use of viral DNA as a vector of genetic elements.

RNA polymerases of mollicutes are insensitive to rifampin, a property shared with RNA polymerases of archaebacteria. However, in subunit structure the mycoplasmal RNA polymerases appear to resemble those of eubacteria. Promoter and terminator regions in the few mycoplasma genes sequenced so far also appear to resemble those of eubacteria. In fact, cloned mycoplasmal genes could be expressed in *E. coli* from their own promoters, indicating that the *E. coli* transcription machinery can recognize the mycoplasmal transcription signals. The ability to express mycoplasmal genes in *E. coli* opens the way for large-scale production of mycoplasmal proteins, facilitating in this way development of vaccines made of specific antigens.

Mycoplasmal ribosomes have attracted particular attention because they are composed of highly conserved molecules useful as phylogenetic markers. Mycoplasmal ribosomes resemble typical eubacterial ribosomes in structure and composition. A significant portion of the *M. capricolum* genome encodes for the 51 ribosomal proteins. Since the mycoplasmal genes for these proteins are rich in A+T (71 mol%) they preferentially use A- and U-rich codons to

produce proteins which resemble very much the corresponding ribosomal *E. coli* proteins, for which the genes have only a 49 mol% A+T content (190).

The mycoplasmal 5S rRNAs are shorter than those of other procaryotes, while the 16S rRNAs contain unique oligonucleotide sequences not found in other procaryotes. There are only one or two gene copies for each rRNA species in the genome of mollicutes. The genes are organized in operons in the typical eubacterial fashion 5'-16S-23S-5S-3'. No tRNA genes were found in the spacer region separating the 16S from the 23S rRNA genes. This region was extremely rich in A+T (80 mol%), a logical outcome in light of the need for a high G+C content in the conserved rRNA genes themselves.

Mycoplasmas contain fewer isoaccepting tRNA species than eubacteria, and the tRNAs are generally poor in modified nucleosides, a property which does not impair their function, even in a heterologous *E. coli* system.

Cloned rRNA genes were found to be useful as general probes for the detection and identification of mycoplasmas, while species-specific protein gene probes show the potential of differentiating between mycoplasmas. There can be little doubt that development of these probes into diagnostic kits will considerably improve the diagnosis of mycoplasma infections.

A variety of viruses and plasmids are found in certain groups of mollicutes, particularly in spiroplasmas and *A. laidlawii*. Viral and plasmid DNA is frequently integrated into the mycoplasmal chromosome, leading to lysogeny in the case of viruses. The significant quantities of free and integrated plasmid DNA found in some mycoplasmas would indicate a possible role for them, but so far nothing has been elucidated. Despite the lack of a cell wall in mycoplasmas their viruses follow, in general, the morphological entities of classical bacteriophages, except for MVL2 and L172, which are enveloped spherical viruses with no capsid structure. The genome of mycoplasma viruses is made of DNA in the circular double-stranded, linear double-stranded, or circular single-stranded conformation. The lack of a cell wall from the host apparently influenced the mode of release of mycoplasma viruses, which resembles that of animal viruses in being continuous and not accompanied by host-cell lysis. The use of mycoplasma viruses and plasmids as vectors for gene transfer is plausible but has yet to be developed.

The peculiar properties of mycoplasmas give a special appeal to studies on their phylogeny. According to one model, mycoplasmas represent the surviving descendants of exceedingly primitive bacteria that preceded the procaryotic-eucaryotic cell split, whereas the second model claims that mycoplasmas arose by degenerative evolution from gram-positive bacteria, more specifically, certain clostridia. While the data obtained from recent sequencing of rRNA and tRNA molecules appear to support the second model, the pronounced phenotypic and genotypic diversity of members of *Mollicutes* and their peculiar and bizarre properties have led to the idea that mycoplasmas constitute a group in a state of rapid evolution. Abstaining from taking sides in the controversy, it appears that as long as bacterial taxonomy will not be based on phylogeny, the mycoplasmas will continue to be considered as a distinct group, easily distinguished phenotypically and genotypically and entitled to at least the status of a class.

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